# Lucerne (*Medicago sativa* L.) establishment after inoculation with different carriers of *Ensifer meliloti* sown on five dates at Lincoln University

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A thesis
submitted in partial fulfilment
of the requirements for the Degree of
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Qakathekile Khumalo

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# Lucerne (*Medicago sativa* L.) establishment after inoculation with different carriers of *Ensifer meliloti* sown on five dates at Lincoln University

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Lucerne (*Medicago sativa* L.) is the highest yielding temperate forage legume, and fixes as much as 360 kg N/ha/year. Its establishment in dryland Canterbury depends on understanding critical factors such as sowing time and inoculation techniques that increase chances of seedling survival and stand persistence. Measurements occurred for a complete growing season after sowing 'Stamina 5' lucerne inoculated with peat, lime coating, ALOSCA ® granules and a bare seed control on 4 October, 4 November, 2 December 2010 and 10 January and 7 February 2011 at Lincoln University Canterbury. No emergence differences were observed from the different inoculants. Coated seed produced a higher initial plant population of 331 plants/$m^2$ which was 68% of the 490 seeds/$m^2$ sown. It also had the highest population 12 months later although seedling numbers had declined by 27%.

Regardless of differences in plant population per treatment, dry matter (DM) yields were not different. Accumulated crop DM yields were however different among sowing dates and decreased with each successive sowing. SD 1 (4 October) gave the highest yield of 15 t/ha whilst SD 5 (10 January) yielded only 2.5 t/ha. Lower DM yields in the later sowing dates were explained by less intercepted light and reduced radiation use efficiency (RUE) probably due to changes in DM partitioning from shoots to roots. Specifically, SD 5 had a longer

phyllochron (65 °Cd per node) than SD 1 (44.6 °Cd/node) at a base temperature of 5 $^{o}$C. No differences were observed in canopy architecture for the different sowing dates as shown by a common extinction coefficient of 0.75. RUE for shoot production ($RUE_{shoot}$) in regrowth crops of all sowing dates was 1.1±0.065 g DM MJ of intercepted photosynthetically active radiation ($PAR_i$). This was 18% higher than the 0.9±0.033 g DM/MJ in seedling crops. Crops from the latest sowing grew 1.84±0.26 kg/ °Cd compared with 3.84±0.22 kg/ °Cd for the earliest sowing. Thermal time accumulation to 50% buds visible ($Tt_{0\text{-}bv}$) decreased from 686 °Cd to 651 °Cd as the photoperiod lengthened in summer from 13 to15 hours for SD 1 and SD 2.

Genotypic characterisation of bacteria isolated from inoculated root nodules and its identification using PCR and 16S methods generated bands for each isolate. These were sequenced and identified to genus level revealing the presence of seven common genotypes. Amongst these, *Rhizobium tibeticum* was the most dominant species with an occurrence of 14.5%. *Ensifer meliloti*, the rhizobia responsible for nodulation and subsequently $N_2$ fixation in lucerne was the second most dominant with an occurrence of 14%. It was also found present in all inoculant carriers characterised but not the bare seed control. These results suggest dominance and effectiveness of resident soil bacteria over introduced *Ensifer meliloti* in nodulating lucerne. The study emphasises the importance of sowing early in spring or summer to take advantage of the longer growing season and the use of the summer fallow to supplement soil moisture for successful lucerne establishment.

**Key words**: alfalfa, flowering, leaf area index, phyllochron, *Sino-rhizobia meliloti*.

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# Chapter 1

# Introduction

Lucerne (*Medicago sativa* L.) is the highest yielding temperate forage legume. It originated in the warm dry climate of Persia in central Asia before spreading to the Mediterranean and into North and South America (Michaud *et al.* 1988). It is now grown throughout the world including New Zealand. Lucerne is a highly productive protein and mineral rich legume suitable for direct grazing or conservation as hay, silage and artificial dehydrated forage. Its high voluntary intake characteristics and nutritive value improve individual livestock performance (Conrad & Klopfenstein 1988). On account of its N fixing abilities, lucerne is also a valuable break crop in organic and arable farming systems.

*Ensifer meliloti* is responsible for nitrogen fixation in lucerne (Frame 2005). It is also known to nodulate sweet clover (*Melilotus* spp), fenugreek (*Trigonella* spp), and other Medicago species. Delivery of inoculant to lucerne seed at sowing is considered important to ensure rhizobial survival between its introduction to the soil and the development of a legume rhizosphere and hence successful nodulation (Wynn-Williams 1982). According to Graham (1992), *E. meliloti* occurrence is reduced below pH 6 and is affected by the presence of mineral N. In most cases, commercial lucerne inoculation is recommended for maximum crop productivity. However recent results for white clover (*Trifolium repens*) suggest that if sufficient effective rhizobia are already present in the soil, perhaps as a result of a previous crop, inoculation may not be necessary (Lowther & Kerr 2011).

The use of rhizobial inoculants in agricultural production aims to ensure that the most effective microsymbiont occupies the largest proportion of nodules formed on the target host legume in the field (Thies *et al.* 2001). Improvements have been made in inoculant formulations and application practices and strains are now selected on the basis of saprophytic competence as well as nitrogen fixation capacity. The time of lucerne sowing in New Zealand is most frequently in spring but autumn sowing is also possible. This range of sowing dates can affect soil moisture conditions at sowing. Sowing into dry soil reduces rhizobial survival and results in soil crusting which consequently leads to poor and uneven emergence (Wynn-Williams 1982).

The overall aim of this study was to quantify the impact of time of sowing and soil surface moisture conditions on seedling establishment and inoculant effectiveness. The success of commercial inoculation may be affected by soil conditions at sowing. The experiment included five sowing dates from late spring to late summer. This created the different moisture regimes and enabled crop growth, development and subsequent yields from each of the treatments to be examined. The specific rhizobia present in the inoculum were genotypically characterized and compared with those found in the root nodules of inoculated plants.

This thesis is presented in seven chapters. Chapter 2 reviews agronomic factors contributing to the successful establishment of lucerne and the physiological mechanisms that control yield formation and crop persistence. This is followed by a detailed outline of the field experiment in Chapter 3 including all measurements taken. The results from the field experiment (agronomy) are presented in Chapter 4 and discussed in Chapter 5. DNA characterisation is described and explained in Chapter 6. All results and their practical implications and the potential for future research are discussed in Chapter 7.

# Chapter 2

# Review of Literature

## 2.1 Lucerne agronomy

Reliable guidelines for seedling establishment are essential for successful lucerne production (Thies *et al.* 2001). Application of lime, based on soil analysis results, is important to raise the soil pH to optimum levels of 5.8-6.0 (White 1967) for nodulation, root development and plant growth. Annual weeds and grass grubs (*Costelytra zealandica* L.) severely depress lucerne yields and must be eliminated prior to cultivation and stand establishment through repeated cultivation (Palmer & Wynn-Williams 1976). Lucerne time of sowing is dependent on soil moisture, soil temperature, weed infestation and pest incidence. Spring sowing (September, October, November) makes use of available soil moisture and gives seedlings a competitive edge over annual winter weeds that emerge later. For successful lucerne nodulation, inoculation with certified viable inoculum is a critical step and should be carried out at the time of sowing. Currently ALOSCA ®, lime coating and peat are the commercial lucerne inoculants available in New Zealand. This review discusses agronomic methods essential for successful lucerne establishment. The aim is to describe agronomic factors that contribute to total and partial failures in establishment of lucerne and identify best seedling management practices.

## 2.2 Yield

In the driest areas of New Zealand, such as the east coast and Central Otago, yields from resident grass dominant pastures are characteristically low (White 1982) and regeneration after drought is slow or absent (Avery *et al.* 2008). Adoption of lucerne and more drought tolerant grass and legume combinations (Burton 1972) that respond rapidly to summer rainfall and convert it into high quality forage is therefore paramount to the success of dryland farming in these regions (Avery *et al.* 2008). Lucerne annual yields vary considerably depending on rainfall and soil water holding capacity. These range from 28 t DM/ha/yr on a Wakanui silt loam soil at Lincoln University under irrigation (Brown *et al.* 2006) to 3 t DM/ha/yr during summer drought in Central Otago (Parle 1967).

Lucerne has also produced over 20 t/ha of DM, 43% more DM annually than pasture under rainfed conditions on rich moisture retentive soils in the North and South Islands of New Zealand (Douglas 1986). Regardless of yield variations arising from drought (Douglas & Kinder 1973), lucerne is superior to the production and persistence of other legumes and grasses under such conditions (Brown *et al.* 2006; White & Meijer 1978). In a five year study of dryland pasture yields in Canterbury, Mills *et al.* (2008) reported consistently higher lucerne DM yields of 13.1-18.5 t/ha/yr which out-yielded the highest grass based pasture by >6.6 t DM/ha/yr. These higher yields enabled lucerne to maintain superiority over grass based pastures particularly during periods of water stress in summer and autumn. This lower sensitivity to moisture stress in lucerne compared with other pasture species was previously observed through a relative increase in lucerne advantage from 25% to 105% as annual rainfall decreased from 700 to 300 mm (Douglas 1986).

More recently, Kearney *et al.* (2010) quantified yield improvements of lucerne, perennial rygrass (*Lolium perenne*) and white clover (*Trifolium repens*) over un-improved browntop (*Agrostis capillaris* ) dominant pasture in relation to rainfall and soil moisture deficits. Lucerne produced 4.2-8.4 t DM/ha compared with 3.1-5.3 t DM/ha for rygrass/white clover and 1.5 t DM/ha for the un-improved pastures over two years. In the first year (2008/2009), lucerne produced 6.1 t DM/ha from 596 mm of rainfall compared with 2.8 t DM/ha of an establishing perennial ryegrass pasture. Soil moisture stored from autumn, winter and spring rainfall affected pasture yields and gave a water use efficiency of ~16.0 kg DM/ha/mm for lucerne compared with 3.5 kg DM/ha/mm for browntop dominant pasture.

## 2.3 Water use efficiency (WUE)

Efficient utilisation of available soil moisture is the most important factor in the eastern regions of New Zealand that are prone to recurring summer droughts (Willems 2006). Moisture supply is the main factor limiting lucerne production throughout the growing season. In New Zealand, lucerne production increases in proportion to the available water holding capacity of soils (Douglas 1986). In dryland systems, the combination of soil available water capacity and the depth of extraction by roots contribute to annual WUE. This was defined by Moot *et al.* (2008) as the ratio of total dry matter accumulation to total water into the system. Brown (2004) defined it as the net photosynthesis of a pasture relative to the amount of water it transpires. Annual WUE can be calculated from measurements of total annual dry matter yield (kg/ha) and total water in the system as illustrated in Equation 1:

**WUE = Y/ (R+I+ASWC-D)** **Equation 1**

Where: Y is total dry matter yield/ha, R is rainfall, I is irrigation (in rainfed systems), ASWC is available soil water content, D is drainage (calculated from water lost from the deepest soil layers in the absence of pasture growth).

Irrigation timing and the number of rainfall events determine the water available for use by pastures and the ability of the soil to store water. For irrigated pastures, management of the balance between water supply and demand can be used to increase WUE (Gandee *et al.* 1999). Moot *et al.* (2008) measured the WUE of dryland perennial ryegrass and lucerne in Canterbury, New Zealand (Figure 2.1). In spring, on a deep Wakanui silt loam, lucerne achieved a high WUE of 40 kg DM/ha/mm from the extraction of 328 mm of water to a depth of at least 2.3 m. Ryegrass in contrast, extracted 243 mm to 1.5 m depth and yielded a lower WUE of 18 kg DM/ha/mm. On a very stony Lismore soil, lucerne extracted 131 mm of stored soil water to the same depth of 2.3 m resulting in a low WUE of 16 kg DM/ha/mm. This was consistent with ryegrass with a water extraction of 129 mm at a depth of 1.5 m. Thus, for optimum utilisation of soil water holding capacity, Moot *et al.* (2008) recommend the establishment of deep rooting species such as lucerne on deep rather than shallow soils. This is vital in maximising extraction of rainfall stored in the soil profile giving greater annual pasture production and WUE. Gandee *et al.* (1999) attributed the improved drought tolerance of lucerne compared with ryegrass/white clover pasture, to a deep root system in excess of 3 m. This was consistent with Douglas (1986) who reported sustained growth for up to two weeks more on lucerne than rygrass/white clover pastures grown on soils with low potential available water content.

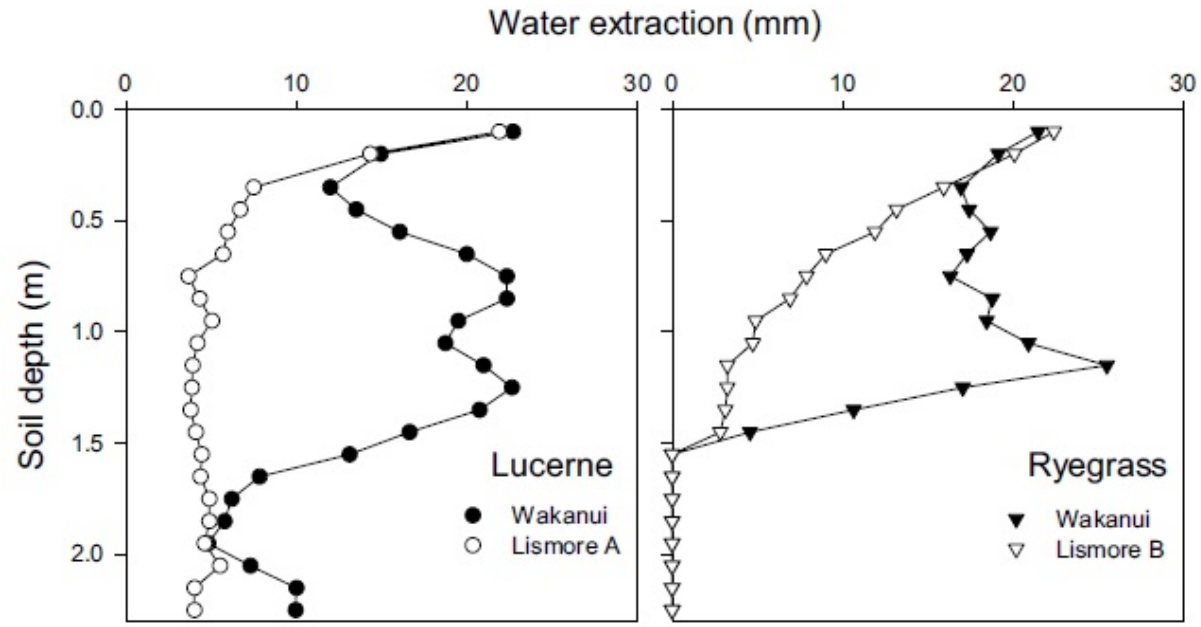


**Figure 2.1 Water extraction (mm) from 0-2.3 m depth for each 0.1 m soil layer for lucerne (circles) and grass based pasture (triangles) on a deep Wakanui silt loam (solid symbols) or a Lismore (A) very stony loam and Lismore (B) stony loam (open symbols) (Moot *et al.* 2008).**

## 2.4 Rhizobia

Rhizobia are aerobic and typically gram-negative, rod shaped motile soil inhabiting bacteria (Spent 2001) that are capable of forming root nodules on legumes (Weir 2006). They are classified into six genera and 57 accepted species most of which are α-Proteobacteria. The genera responsible for N fixation in lucerne is *Ensifer meliloti* (Frame 2005), formerly known as *Sinorhizobium meliloti* (Young 1996). *Ensifer* does not occur naturally in New Zealand soils hence the need to inoculate seed for successful lucerne establishment (Thies *et al.* 2001).

Parle *et al.* (1973) investigated *E. meliloti* survival and persistence on lucerne seed inoculated by conventional methods and observed a rapid decline in recoverable rhizobia from 10 000 counts/seed to a few hundred an hour after inoculation. This was ascribed to the rapid death of rhizobia due to a lack of protection during drying suggesting that failed lucerne establishment was a result of poor rhizobial survival on the seed (Parle *et al.* 1973). According to Blair (1971), 65% of lucerne established had 30% or less plants nodulated 6-8 weeks after sowing. He attributed this to low soil pH at the time of sowing stating that rhizobial nodulation required a soil pH of 5.8-6.0. At a soil pH below 4, the soil is usually too acidic and contains high aluminium unfavourable for *E. meliloti* survival. As the pH starts to increase above 4.5, conditions become more favourable and aluminium toxicity is reduced (Moir & Moot 2010).

Nodulation reaches its peak at pH 5.8-6.0 for most strains after which the soil becomes too alkaline and rhizobial survival becomes compromised.

## 2.5 Inoculation

Legume inoculation is a significant agent for improving crop productivity and soil fertility when used where needed and if it performs as expected (Selander *et al.* 1986). It is aimed at maximizing survival of inoculant between its introduction to the soil and the development of a legume rhizosphere which it can colonize. Inoculation is important because many soils used for legume cultivation may not contain adequate numbers of highly effective rhizobia, or may contain high numbers of ineffective or partially effective strains. If sufficient infective rhizobia are already present in the soil, perhaps as a result of repeated cultivation of a particular crop in the same area, inoculation may not be necessary.

Successful inoculation requires large numbers of viable rhizobia per seed to ensure effective nodulation. According to the Australian Legume Inoculants Research Unit (ALIRU), a minimum of 1000 effective rhizobia are required per seed for effective nodulation (Thies *et al.* 2001). Parle *et al.* (1973) argue that high viable rhizobial counts in inoculants do not always lead to satisfactory counts on inoculated seed. This is consistent with Blair (1971) who stated that survival of viable rhizobia on seed was of far greater importance than their quantity. Lack of competitiveness is a major cause of nodulation failure when new strains from ecologically different sites are introduced into soils with high numbers of indigenous strains (Triplett & Sadowsky 1992). These highly competitive indigenous rhizobial populations impede establishment of commercial strains resulting in unsatisfactory levels of colonization in the year of inoculation (Bromfield *et al.* 1986).

In the United Kingdom, Gandee *et al.* (1999) observed adequate nodulation and growth from inoculated and un-inoculated lucerne grown in soils rich in *E. Meliloti.* They observed no differences in DM and concluded that inoculation may not always be necessary in lucerne establishment. Inoculation therefore serves as a form of insurance due to the absence of simple indicators of the need for inoculation (Thies *et al.* 2001). Lowther & Kerr (2011) recently examined the need to inoculate white clover in New Zealand. Like lucerne, white clover inoculating rhizobia were absent in New Zealand prior to European settlement. Over time they have spread widely throughout the country and are now resident in high levels capable of effective nodulation. Similarly, *E. Meliloti* populations may have survived and

persisted throughout the years making it unnecessary to inoculate seed at sowing in some situations. Further research is required to ascertain resident levels of *E. Meliloti* in the different soil types and regions and quantify the effectiveness of viable strains found.

## 2.6 Inoculum

Inoculants are produced in a carrier material which may be added directly to the seed or placed in the furrow prior to sowing. Yardin *et al.* (2000) suggested that the carrier substrate is a critical part of the product formulation and must be capable of supporting high numbers of the intended microbes. Essential characteristics of a suitable carrier include; a high organic matter content (>60%), low soluble salt content (<1%), high moisture holding capacity (150-200%) and the presence of nutritive media for rhizobial growth and survival on inoculated seed (Richardson *et al.* 1986).

### 2.6.1 Peat inoculum

Peat is the most commonly used carrier for rhizobial inoculants mainly because of its high moisture holding capacity and dual ability to foster multiplication of rhizobia and protect it once applied to the seed coat (Herridge *et al.* 2002). Peat-based inoculants are thought to give a considerable measure of protection to the rhizobia on the seed surface (Parle *et al.* 1973).

Sterile and non-sterile peats are common carriers for rhizobia although sterile peats are generally preferred by farmers because they contain up to 100 fold more rhizobia than non-sterile types (Thompson 1983). They are however costly, easily contaminated, require strict asceptic maintenance and suffer consistent unsatisfactory nodulation of new stands particularly when dry weather prevails (Wynn-Williams 1976). Peat inoculum reduces rhizospheric pH due to the acidic nature of most peat deposits (Roughley 1970). Pre-mixing peat with seed prior to sowing is laborious and time consuming especially when establishing large fields (Werner *et al.* 2005).

### 2.6.2 Lime coating

Other common alternative inoculant carriers include coating or pelletizing seed. Coating lucerne seed is beneficial because nodule bacteria are in close proximity to the roots of the

germinating seedling and can therefore rapidly produce effective nodules. The main coating component is lime which corrects soil pH and offers viable bacteria protection from stress and desiccation which can rapidly decrease rhizobial populations. Manufacturers' commercial claims state that coated seed is ballistic and weighs approximately 1.6 times more than uncoated seed. This enables greater ground penetration and increased seed to soil contact. Its size is convenient and allows greater control of the sowing rate and seed distribution.

According to Horikawa & Ohtsuka (1996), lime coated lucerne seed experiences a rapid degree of nodulation and early seedling growth compared with peat inoculated seed. Coated lucerne forage yields were also reported to be 85% higher than peat inoculated treatments in the first year and 47% higher in the second year (Rice & Olsen 1988). These results are consistent with those of Horikawa & Ohtsuka (1996) who observed a 30% increase in yield in the first and second years. Due to early and rapid nodule formation, coated seed had a higher survival rate and vigorous plant growth in the first year and up-to the first harvest of the second year (Horikawa & Ohtsuka 1996).

### 2.6.3 ALOSCA ®

ALOSCA ® technology is based on a bentonite clay granule that contains high numbers of viable rhizobial cells. It is considered by its manufacturers to be far more practical for farmers than peat based inoculants which require refrigeration at 4 °C prior to planting. Commercial claims for ALOSCA ® are that it is highly suited to shallow sowing, buffers bacteria against the harmful effects of pesticide seed dressings and increases yields and nitrogen fixation in pasture and legume crops by at least 50% (Kiwiseed 2010). Its granules can be applied as a mix with the seed or with the fertilizer at seeding. They are reported to stay in the ground when conditions are either dry or wet for extended periods without losing viability. If dry conditions prevail following seeding, the clay reportedly "closes up around the microbes to maintain viability".

## 2.7 Fertilizer/nutrient requirements

### 2.7.1 Soil pH and micronutrients

Soil pH serves as a guide to where lucerne should be grown by indicating the potential presence of soluble aluminium (Al) in soils (Douglas *et al.* 1987). White (1967) recommends

a minimal soil pH of 5.8-6.0 as the ideal range for successful nodulation and establishment of lucerne. Optimum pH varies with soil texture, organic matter and soil chemical properties (McLean 1971). Lime is recommended prior to stand establishment to offset soil acidity based on soil test results. By raising soil pH, lime increases survival and multiplication of rhizobia and improves root development and plant growth, possibly by reducing manganese (Mn) and Al toxicity in the plant and soil (White 1967). According to Moir *et al.* (2000), legume establishment and maintenance are retarded by low soil pH and low available phosphorus (P) and sulphur (S), a common condition in 500 000 ha of farmed high country soils in New Zealand. Lucerne is specifically intolerant of acidic soil conditions and related Al and Mn toxicities (Su & Evans 1996).

In a study of the effects of liming at rates of 2-8 t/ha on a high country brown stony soil in the Lees Valley in Northern Canterbury, Moir & Moot (2010) reported soil pH changes of 0.15 units per tonne of lime applied in the 0-75 mm soil horizon. Soil exchangeable Al dropped to <0.3 me/100g in the 0-75 mm soil horizon and from 0.9 to 0.1-0.2 me/100 g in the surface soil horizon, clearly demonstrating the ability of low liming rates to reduce exchangeable Al to safe levels. Lucerne yields were consistent at 0.7-1.2 t DM/ha for all rates of lime applied suggesting the role of soil depth and hence plant available water in limiting yield rather than soil pH or Al levels. The amendment of soil fertility with appropriate fertilizer usually occurs based on a soil test 6-8 months prior to sowing.

### 2.7.2 Macronutrients

The most important nutrients for stand establishment are Phosphorus (P) and Potassium (K) (Wynn-Williams 1982).

#### *2.7.2.1 Phosphorus (P)*

P is particularly important because of its role in root development of seedlings (Abdolzadeh *et al.* 2010) and its involvement in adenosine triphosphate (ATP) which is associated with nitrogenase activity (Hawkesford *et al.* 2012). P is often band applied at sowing below the seed at rates based on soil test results (Jensen 1941). This was however reported to promote weed growth by Stephen (1970) who in turn suggested the application of phosphorus based fertilizers during periods of vigorous lucerne growth.

#### *2.7.2.2 Potassium (K)*

Adequate K in lucerne is critical in the conversion of amino acids to proteins and transportation of photosynthates from source to sink (Collins & Duke 1981). In most cases it is not required for lucerne establishment unless deficient as a result of 'cut and carry' from paddocks (Douglas 1986).

#### *2.7.2.3 Nitrogen (N)*

N is generally not necessary in lucerne establishment (Lee & Smith 1971). According to Bailey (1983), N content in lucerne increases with K availability. A study by Ward & Blaser (1961) reported that increasing N rates from 0-90 kg/ha reduced seedling numbers and showed no improvement in seedling growth. This was attributed to the inhibitory effect of available soil N on $N_2$ fixation, soil nitrate and to a larger extent, ammonium, whose degree of inhibition increases with concentration (Munns 1967).

#### *2.7.2.4 Sulphur (S)*

S is deficient in many regions of New Zealand. It is required in plants with N for protein synthesis and for vigorous growth of lucerne and $N_2$ fixation (Ludecke 1967).

### 2.7.3 Micronutrients

In New Zealand, Molybdenum (Mo) is the most important trace element deficiency of legumes (Sherrell 1984). It is routinely applied to lucerne grown on coarse water sorted pumice soils of the North Island and all soils in the South Island excluding brown-grey earths and recent soils that are not strongly leached (Douglas 1986). In lucerne, Mo is required for $N_2$ fixation and N assimilation into proteins. Deficiencies are common on acid soils below pH 5 and can be corrected by liming (Vinuesa *et al.* 1998). Lucerne is also sensitive to Boron (B) availability and responds to its application more than red clover (*Trifolium pratense* L.) and timothy (*Phyleum pratense* L) (Mullis *et al.* 1987). B deficiency is observed during drought and at pH 6 or above. It is associated with yellowing of the top leaves and shortening of upper internodes (Crocker *et al.* 1985).

## 2.8 Soil preparation

Satisfactory stands can be established using non-till drills, such as the Øyjord cone seeder, that are adapted to shallow and accurate seed placement (Burton 1972). Firming the seedbed through rolling prior to sowing improves emergence under moisture limiting conditions.

## 2.9 Sowing depth and moisture availability

An ideal seedbed for lucerne and all herbage species should be moist, firm, sufficiently fine and granular to maximise seed to soil contact (Wynn-Williams 1982). Depth of seed placement at sowing is dependent on soil type and moisture availability. Stanley *et al.* (2002) recommend 5-15 mm depths on heavy clay-loams compared with up to 25 mm on sandy soils. In New Zealand, lucerne is generally sown in spring or early summer when the gravimetric soil moisture content is adequate for rapid germination and emergence of seed (Wynn-Williams 1982). Germination and seedling emergence depend on availability of soil moisture and humidity around the seed. Poor germination is often a result of inadequate soil moisture which causes seedling death through desiccation (Campbell & Swain 1973). Dry sowing also causes reduced rhizobial survival and increases soil crusting with a consequence of poor seedling emergence (Wynn-Williams 1982).

## 2.10 Temperature

Successful lucerne establishment requires rapid germination and emergence (Whitelaw 1975). Thermal time (Tt) is a common approach used to express the relationship between temperature and plant development between two growth stages (Arnold & Monteith 1974) such as sowing to emergence. It is calculated as the mean temperature minus the base temperature (Tb) below which no development can occur (Equation 2).

$$Tt = \sum \left( \frac{Tmax + Tmin}{2} \right) - Tb \qquad \textbf{Equation 2}$$

According to Wynn-Williams (1976), the rate of lucerne germination increases with temperature. When moisture is adequate, seedling survival and establishment are highest in October and November and lowest in December when soil temperatures are super optimal

(Figure 3.2b). The most rapid rate of lucerne germination was observed at 5-30 $^{o}$C (McWilliam *et al.* 1970). Establishment of over-sown lucerne and white clover was examined by Musgrave (1977) who found it to be optimum at 3-7 $^{o}$C soil temperatures at 0.1 m depths. Lucerne base temperature (Tb) and thermal time (Tt) requirements for germination were defined as 0.9 $^{o}$C and 39 $^{o}$Cd, respectively (Moot *et al.* 2000).

## 2.11 Seeding rates

In New Zealand recommended seeding rates range from 8 to 10 kg/ha or 300-500 seeds/m$^2$ (Palmer & Wynn-Williams 1976). In spring, 2.5 kg/ha is adequate under ideal seedbed conditions whilst in autumn a minimum of 10 kg/ha is required under marginal conditions in the absence of pests and diseases (Burton 1972). During the seedling year and into the first production year, higher seeding rates may give higher crop yields, but not in proportion to the increased seed rates. Optimum seeding rates in lucerne pasture should be related to sowing date and are determined by the amount and distribution of rainfall, sowing method, seedbed preparation, soil type and tilth (Burton 1972). After the first year, only 40 to 50% (Thies *et al.* 2001) of seedlings sown survive as a result of competition, frost, pests and diseases.

According to Palmer & Wynn-Williams (1976), yields from established stands reached a maximum with plant densities as low as 30 plants/m$^2$. This suggests that, provided the sowing rate was sufficient to give 30 plants/m$^2$ in the following season, then stand life is independent of the initial sowing rate (Figure 2.2). 'Wairau' lucerne was sown at 5.6, 11.2 and 16.8 kg/ha in an experiment to establish lucerne density per unit area and survival over an eight year period. Plant density declined in all populations, showing a higher death rate in the denser treatments; 11.2 and 16.8 kg/ha compared with 5.6 kg/ha. Plant population in the lowest sowing rate treatment (5.6 kg/ha), remained constant in the last three years while the other treatments continued to decline towards an asymptote. The death rate appeared to be population dependent and stands were noted to exhibit self thinning over time to an equilibrium population of 40-50 plants/m$^2$ which was above the minimum required for maximum production (Wynn-Williams 1982). Teixeira *et al*. (2007a) recently examined the plasticity in lucerne yield components as stand population declined. They noted an increase in shoot number from 6 to 13 as the plant population declined from 130 to 60 plants/m$^2$.

**Figure 2.2 Seeding rate; 16.8 kg/ha (● ), 11.2 kg/ha (■) and 5.6 kg/ha (▼) and lucerne density over time (Palmer & Wynn-Williams 1976).**

## 2.12 Management

Lucerne seedling management involves control of grazing, weeds and insects.

### 2.12.1 Grazing

New lucerne stands are commonly grazed at about 150 mm height when the roots are 250-260 mm deep to reduce weed competition and stimulate regrowth (Iversen 1967). This is consistent with reports by Musgrave (1972) who observed that a quick early grazing at the 4-5 leaf stage was important in reducing weed competition and had no effect on subsequent yields if done leniently (Wynn-Williams 1982). A bold heavy grazing, according to Janson (1972), resulted in a substantial reduction in lucerne yields in the second year.

### 2.12.2 Weed control

Winter annual weeds and perennials grow and fill all available gaps left after lucerne has self thinned and gone dormant. They can produce seed heads which reduce pelt values and interfere with haymaking and reduce hay quality (Palmer 1982). Despite this, winter annuals also increase production of high quality feed without reducing lucerne production in the stands in which they grow (Palmer & Wynn-Williams 1976).

Palmer & Wynn-Williams (1976) found no permanent benefit of spraying out winter annuals beyond the first spring citing a reduction rather than an increase in spring available feed in addition to the cost of spraying and the limited weed suppression achieved. In contrast, perennial weeds such as browntop (*Agrostis capillaris*), docks (*Rummex* spp), and horehound (*Marrubium vulgare*) were reported to severely depress lucerne yields unless eliminated through herbicide spraying (Palmer & Wynn-Williams 1976), grazing management, improved soil fertility and drainage (Stephen 1964). Alternative annual winter weed control strategies include; early spring establishment at lower temperatures to reduce competition from slower establishing winter annual weeds and spraying to reduce seed damage to pelts (Palmer 1982).

### 2.12.3 Insects

A number of insect species feed on lucerne and are capable of causing severe damage to the crop. Three species of pests that are particularly notorious at establishment in New Zealand include; the grass grub, the blue green lucerne aphid (*Acyrthosiphon kondoi* S.) and the sitona weevil. Grass grub larvae are the most serious root feeding beetles in New Zealand and attack lucerne seedlings up to 6 months old. The adults also cause considerable damage, though of minor significance, by feeding on foliage. A deep thorough cultivation intended to kill grass grub larvae prior to sowing is a common cultural control method (Wynn-Williams 1982).

The blue green lucerne aphid damages lucerne by; stunting plants curling leaves, causing chlorosis and eventually dropping leaves prematurely (Manglitz & Ratcliffe 1988). Kain & Trought (1982) recommended that stands be sown early in spring when temperatures are low (10-15 $^{o}$C) and ideal for aphid survival and immediately sprayed post emergence for effective control. Sitona weevil adults cause near complete defoliation of foliage characterised by notching leaf margins (Kain & Trought 1982). In the South Island of New Zealand, damage generally occurs in mid-summer during periods of slow growth. Seedlings are only susceptible to attack if sown in autumn. Low levels of insecticide sprays in autumn, before egg laying have been used to temporarily relieve adult weevil populations (Kain & Trought 1982). Introduction of *Microctonus aethiopoides*, a parasitoid biological control agent, resulted in ~60% sitona parasitism prior to the bulk of weevil seasonal egg-laying (Goldson *et al.* 2005). This subsequently led to the virtual eradication of sitona as a lucerne pest in New Zealand (Barlow & Goldson 1993).

## 2.13 Biophysical factors affecting lucerne establishment

Development of sustainable best management strategies for lucerne production depends on understanding the physiological mechanisms that control yield formation and crop persistence (Fick *et al.* 1988). Crop biomass is a linear function of accumulated intercepted photosynthetically active radiation ($PAR_i$) over time. Light interception is modulated by canopy expansion through the rate of leaf appearance and leaf area expansion (Robertson *et al.* 2002).

Lucerne performance during the seedling phase influences crop productivity and stand persistence and is a key determinant of plant establishment (Fick *et al.* 1988). Biomass accumulation during the seedling phase represents a large proportion of the annual production in farming systems that use lucerne for short periods, such as for inter-cropping in Australia (Angus *et al.* 2000). There is, however, limited information available on crop physiological responses during this stage, particularly during reproductive development. For instance the time of flowering in lucerne is often used as the basis for formulating agronomic advice in relation to time of defoliation (Wynn-Williams 1982). Also, model parameterization for lucerne phenology is often derived from controlled environment studies due to the absence of comparative field measurements (Teixeira *et al.* 2011).

### 2.13.1 Light interception

According to Teixeira *et al.* (2011) seedlings had a 40% slower leaf area expansion rate (LAER) than regrowth crops (Figure 2.3). This reduction in LAER for seedlings was partially explained by a 34% longer phyllochron (Table 2.1) with a consequent reduction in leaf appearance rates (LAR). This was consistent with a 40% longer phyllochron for seedlings than for regrowth crops reported for ‘Moapa’ and ‘Vernal’ lucerne cultivars grown under controlled environments (Pearson & Hunt 1972). Teixeira *et al.* (2011) observed a consistent phyllochron of 35 $^{o}$Cd at a base temperature of 0 $^{o}$C for regrowth crops (Table 2.1), with photoperiods >12.5 h (Teixeira *et al.* 2007a) but this lengthened during autumn (Brown *et al.* 2005).

### *2.13.1.1 Leaf appearance*

According to Teixeira *et al.* (2011), the longer phyllochron during the seedling stage is an indication that factors other than temperature, control primary leaf appearance (Figure 2.3). A possible explanation is the strong carbon sink competition between root formation and leaf appearance in seedlings, against a limited carbon supply (Teixeira *et al.* 2011). Brown *et al.* (2005) suggested that the limited expression of node appearance and consequently longer phyllochron observed in autumn was a result of regrowth lucerne preferentially allocating assimilates to accumulate vegetative storage proteins and replenish carbon and nitrogen reserves in roots (Avice *et al.* 2003). Similarly, lucerne seedlings diminish nitrogen availability through formation of a root system but lack sufficient rhizobial nodulation to meet nitrogen demand (Teixeira *et al.* 2011).

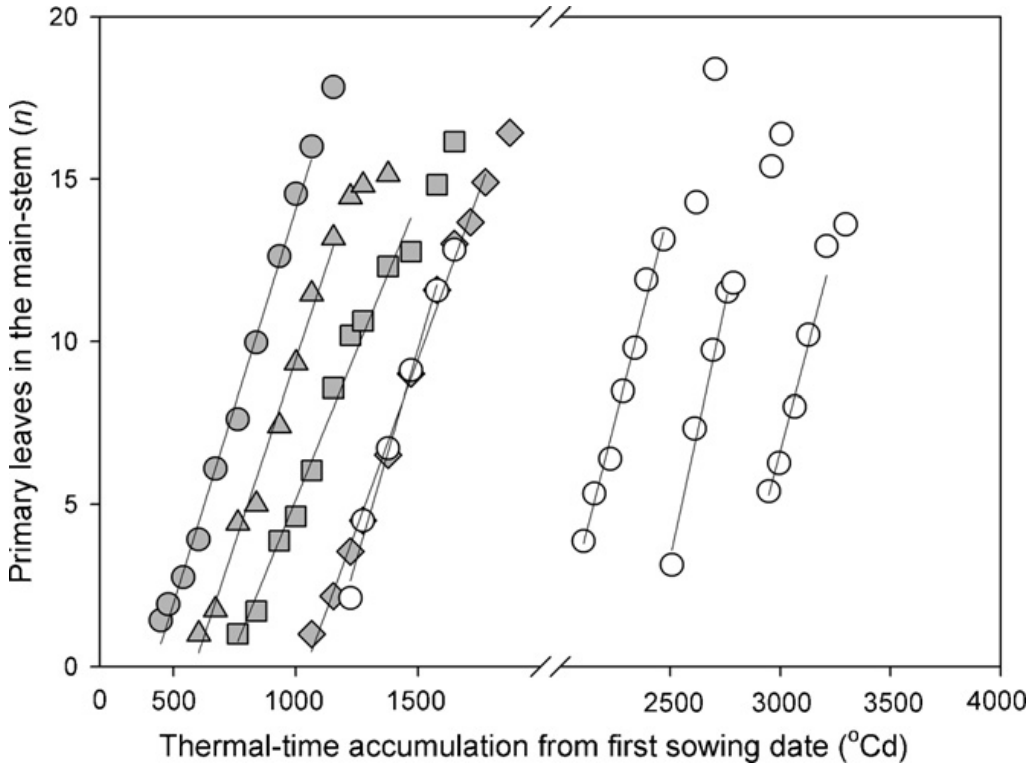


**Figure 2.3 The number (n) of primary leaves per main stem against thermal time accumulation ($Tt_b$ =0°C) after emergence for seedling (closed symbols) and regrowth (open symbols) 'Grasslands Kaituna' lucerne crops sown on 24 October (●), 15 October (▲), 05 December (■) and 27 December (◆) at Lincoln University, New Zealand 2010 (Teixeira *et al.* 2011).**

Teixeira *et al.* (2011) also suggested an ontogenic related decline in the phyllochron as plant development progressed and a lower sensitivity to environmental stimuli to be possible causes of low seedling leaf appearance rates and additional thermal time requirement to reach 50% buds visible stage. The longer phyllochron in seedlings was only partially responsible for the

slower LAER, that was reduced twice as much as leaf appearance (Table 2.1). Teixeira *et al.* (2007b) attributed this reduction in LAER to the existence of smaller leaves that ultimately limited the supply of taproot nitrogen to shoots during early lucerne regrowth and reduced LAI development by delaying recovery of photosynthetic capability and nitrogen uptake.

**Table 2.1 Calculated physiological parameters for seedling and regrowth 'Grasslands Kaituna' lucerne crops grown at Lincoln University, Canterbury, New Zealand (Teixeira *et al.* 2011).**

| Physiological variable | Seedling crops | Regrowth crops |
|---|---|---|
| Phyllochron ($^{o}$Cd leaf$^{-1}$)* | 47 ± 2.3 | 35 ± 1.8 |
| LAER (LAI.$^{o}$Cd$^{-1}$)* | 0.009 ± 0.001 | 0.016 ± 0.001 |
| Extinction coefficient (k) | 0.96 ± 0.008 | 0.89 ± 0.005 |
| $RUE_{shoot}$ (g DM.MJ $PAR_i^{-1}$)* | 1.2 ± 0.16 | 1.9 ± 0.24 |
| Fractional $RUE_{shoot}$ (normalized[a])* | 0.6 ± 0.12 | 1.0 ± 0.15 |

[a] $RUE_{shoot}$ normalized by mean air temperature.

* Means significantly different at $\alpha = 0.05$.

#### *2.13.1.2 Canopy architecture*

Canopy architecture, quantified by the extinction coefficient (k) was unaffected by environmental signals, defoliation frequency or the level of endogenous reserves (Teixeira *et al.* 2007b). Teixeira *et al.* (2011) found no differences in canopy architecture between seedlings and regrowth crops which in addition, achieved 95% light interception, at the same critical leaf area index ($LAI_{crit}$), of 3.2.

### 2.13.2 Conversion of light into shoot biomass

Teixeira *et al.* (2011) estimated the biomass partitioning to roots ($p_{root}$) by calculating the quotient between the radiation use efficiency (RUE) for shoot production ($RUE_{shoot}$) and $RUE_{total}$ and assuming the optimum RUE for total biomass production ($RUE_{total}$) as a constant (Khaiti & Lemaire 1992) further adjusted by mean air temperature (Brown *et al.* 2006). The partitioning to roots yielded 57±0.7% for seedlings and 28±1.2% for regrowth crops. The relatively lower variability in $p_{root}$ for seedlings suggests that environmental factors had less influence on biomass partitioning during this period. In contrast, there is a strong seasonal

response of biomass partitioning to roots observed during regrowth of crops (Teixeira *et al.* 2008). Alternatively, the limited $RUE_{shoot}$ in seedlings could be due to a lower leaf photosynthetic capacity (Teixeira *et al.* 2011) due to limited nitrogen supply to shoots (Avice *et al.* 1997).

Photosynthesis during the regrowth phase was sink limited and source limited in the seedling stage (Baysdorfer & Bassham 1985). Nodule development in seedlings may be hindered by low rates of carbohydrate translocation from immature leaves while leaf growth and increased translocation may be limited by the rate of nodule development. Baysdorfer & Bassham (1985) further suggested that source limitation could be ascribed to the low photosynthetic rate or export capacity of immature leaves if the shift from source to sink limitation in lucerne seedlings occurred as a result of an increased percentage of mature exporting leaves.

### 2.13.3 Photoperiod response

The photoperiod separating optimal from non optimal photoperiods is the minimum optimum photoperiod. For a long day species like lucerne, long photoperiods are optimal and as they shorten, they become non-optimal and flowering is delayed. In non optimal photoperiods there is a delay of flowering that is proportional to the length of the photoperiod. The delay is the photoperiod induced phase (PIP). Photoperiod sensitivity can be described by a photoperiod response model (Figure 2.4) quantified as the slope of the change in flowering per unit increase of photoperiod (Major 1980). The photoperiod response model (Figure 2.4) involves determination of three characteristics: the basic vegetative phase (BVP), maximal optimum photoperiod (MOP) and the slope of the line, which initiates photoperiod sensitivity. The BVP is a period of juvenility, independent of photoperiod, through which the genotype must pass before flowering can be initiated.

**Figure 2.4 Generalized photoperiod response of a quantitative long-day plant (Major *et al.* 1991).**

The physiological mechanisms controlling changes in DM partitioning to lucerne roots are unclear but may be a direct response to short photoperiods and/or low temperatures (Teixeira *et al.* 2008) and consequent changes in source/sink relationships (Teixeira *et al.* 2007c). Noquet *et al.* (2003) suggested that environmental stimuli in autumn may activate genes responsible for the preferential allocation of assimilates into reserve compounds. Short day exposure induced stimulation (Noquet *et al.* 2001) and accumulation (Noquet *et al.* 2003) of β amylase transcript.

A general stability of photoperiod sensitivity rate (Ps) was observed among lucerne genotypes. In 'Grasslands Kaituna' lucerne seedling and regrowth crops, $Tt_{0\text{-}bv}$ increased at a Ps of 106 $^{o}$Cd/h for both crops at photoperiods shorter than the $Pp_{crit}$ of 14 h (Teixeira *et al.* 2011). Major *et al.* (1991) recalculated a rate of 126 $^{o}$Cd/h for different cultivars at a $Pp_{crit}$ of 18 h during the seedling phase. Teixeira *et al.* (2011) plotted combined data sets with Major *et al.* (1991) and observed that the $Tt_{0\text{-}fl}$ model for seedlings intercepts $Tt_{BVP}$ at a similar $Pp_{crit}$ of 18 h (Figure 2.5). This suggested a consistency between seedlings of diverse cultivars and growth conditions. The implication was that the juvenile period may be a consequence of the difference in $P_{crit}$ between seedlings and regrowth crops. As a result, $Tt_{juv}$ became nil at long photoperiods, e.g. $Pp_{crit}$ >18 h (Teixeira *et al.* 2011).

### 2.13.4 Bud visible stage and flowering

Both seedling and regrowth crops had a consistent minimum thermal time requirement of 530 $^{o}$Cd to reach flowering ($Tt_{0\text{-}fl}$). This delimited a common basic vegetative period ($Tt_{BVP}$) for flowering in a diverse group of lucerne cultivars. At photoperiod's shorter than18 h, seedlings increasingly required more Tt to reach flowering. For instance, at a phyllochron of 47 $^{o}$Cd in seedlings and 35 $^{o}$Cd in regrowth crops (Table 2.1), a minimum of ~11 and 15 leaves would appear before floral development, respectively. At a Pp of 15.5 h, ~17 leaves would appear in seedlings in comparison with the minimum of 15 in regrowth crops (Teixeira *et al.* 2011). These values closely compare with 17–21 leaves for seedlings and 13–14 leaves for regrowth crops observed in lucerne 'Moapa' and 'Vernal' grown under controlled conditions (Pp = 15.5 h) (Pearson & Hunt 1972).

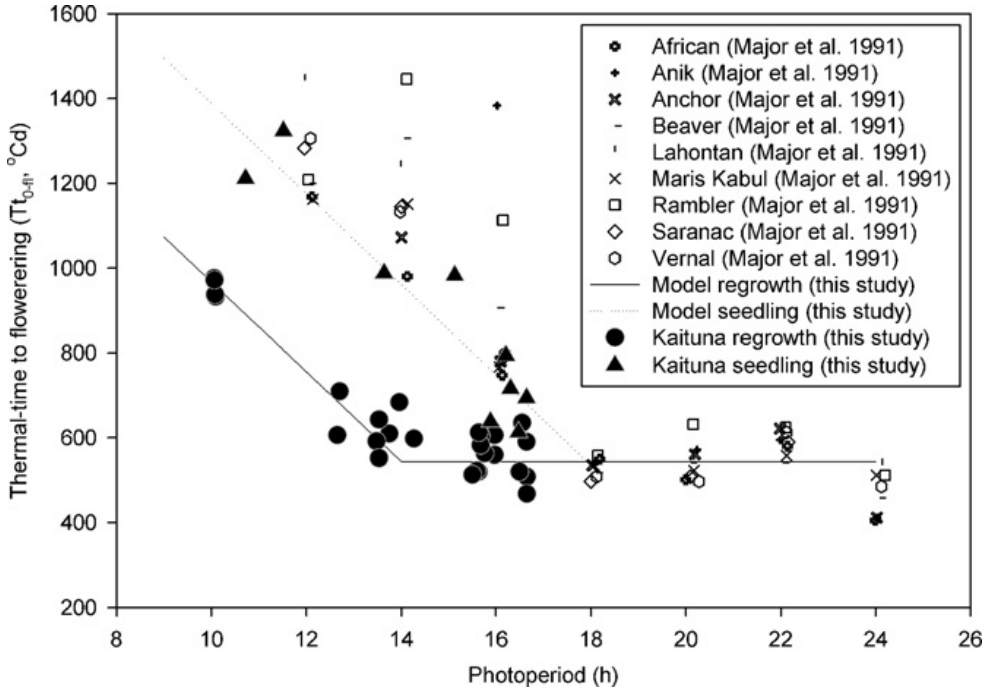


**Figure 2.5 Thermal time requirement for lucerne flowering ($Tt_{0\text{-}fl}$) in relation to photoperiod comparing current results with re-analysis of nine different lucerne cultivars. Adapted from Major *et al.* (1991). Note: The $Tt_{0\text{-}fl}$ values for the re-analysed data set were obtained by converting values from "days" at 25$^{o}$C in a growth chamber to thermal time assuming accumulation of 20 $^{o}$Cd/day (Teixeira *et al.* 2011).**

## 2.14 Conclusions

In dryland areas of New Zealand, the factor most limiting reliable lucerne establishment is moisture stress. Sowing time and inoculation techniques also need critical attention to synchronise sowing with periods of high gravimetric soil moisture content. However this is not always possible in areas with variable rainfall so different sowing dates can be used to create different soil moisture conditions and variable biophysical conditions at sowing. These can also be used to see if relationships such as phyllochron, dry matter production and reproductive development can be unified for seedling and regrowth crops and determine if they affect nodulation. It is also worth noting that, inoculation is the surest way of delivering *E. meliloti* into lucerne stands in the absence of simple indicators for the presence or absence of nodulating bacteria in a particular soil. The efficiency of delivery from different commercial products has received little research attention.

Reduced light interception and low efficiency of light conversion into shoot biomass are possible explanations for lower yield in seedlings. The rate of reproductive development was delayed at short photoperiods in both seedling and regrowth crops suggesting that temperature was not a driving factor. Lucerne management is ultimately affected by the different patterns of seedling and regrowth development, particularly harvest time, weed control and livestock grazing management. Thus an understanding of the physiological processes at the seedling stage is required to allow best management practices to be developed. These probably require establishment of a root system and root nodules that act as N sources for the regrowth crops whose physiological processes appear to occur at much faster rates.

# Chapter 3

# Materials and Methods

## 3.1 Experimental site

This experiment was located at the Lincoln University Field Service Centre, Canterbury, New Zealand (43°38'S and 172°28'E, 11 m a.s.l) within a 0.98 hectare (140 x 70 m) area of flat land in Block 12 of Iversen Field. The paddock had a history of lucerne/ryegrass from 2004-2007, brassicas in 2008 and a short rotation ryegrass from 2009-2010. The soil is a Wakanui silt loam (*Udic Ustochrept*, USDA Soil Taxonomy) with 1.8-3.5 m of fine textured material overlying gravels (Cox 1978). It was deep ploughed on the 1$^{st}$ of September 2010 after which 20% Sulphur Super (N, P, K, S) was applied at a rate of 20 kg/ha P and 50 kg/ha S following soil test results (Table 3.1) and prior to the first sowing. The field was then deep ploughed, harrowed and rolled and boom sprayed with Treflan® E.C trifluralin (5%) at 3 *l*/ha for weed control. The outer boundaries and buffers of the experiment were pegged and the 80 plots marked out. Germination tests incubated three replicates of 50 bare and coated seeds each at 20 $^{o}$C for at least 36 hours. Germination rates were over 92% for bare and coated seed. A consistent seeding rate of 10.5 kg/ha (30.9 g/plot) and 17 kg/ha (50 g/plot) was established for bare and coated seed, respectively (Table 3.2). An Øyjord cone seeder was used to sow 14 rows per plot 0.15 m apart that were 4.2 m wide and 7 m long with 0.5 m gaps between plots.

## 3.2 Experimental design

A split plot randomised complete block design was used with five sowing dates (SD 1=4 October, SD 2=4 November, SD 3=2 December 2010 and SD 4=10 January and SD 5=7 February 2011) as the main plots. The four seed treatments namely peat, lime coating, ALOSCA ® granules and a bare seed control were sown as subplots in four replicates.

## 3.3 Soil testing

Soil samples were also collected prior to each sowing to determine the macronutrient status. Samples consisted of a bulked sample of 16 soil cores taken from depths of 0-0.15 m across the entire field. Soil $NO_3^-$ and $NH_4^+$ concentration were determined at each sowing through soil analysis by 2M KCl extraction followed by Cadmium reduction and N-(1-Naphthyl)–ethylendiamindinydro-chlorid (NED) colorimetry for $NO_3^-$and 2M KCl extraction followed by Berthelot colorimetry for $NH_4^+$.

**Table 3.1 Soil analysis report taken prior to sowing 'Stamina 5' lucerne on five dates at Lincoln University, Canterbury 2010. Optimum range represents most favourable level for pasture production.**

| | | Soil sampling dates | | | | | |
|---|---|---|---|---|---|---|---|
| | | | 2010 | | | 2011 | |
| **Analysis** | **Unit** | **14/09** | **9/10** | **2/11** | **8/12** | **9/01** | **Optimum range** |
| pH | pH units | 6 | 5.8 | 5.7 | 5.5 | 5.5 | 5.5 - 6.5 |
| Olsen phosphorus | me/100g | 17 | 17 | 20 | 19 | 19 | 25 - 30 |
| Potassium | me/100g | 0.7 | 0.44 | 0.53 | 0.64 | 0.53 | 0.30 – 1.00 |
| CEC | me/100g | 17 | 14 | 15 | 16 | 14 | 12 – 25 |
| Total base saturation | % | 64 | 63 | 63 | 62 | 65 | 50 – 85 |
| Sulphate sulphur | mg/kg | 3 | - | - | - | - | 7 – 15 |
| Total nitrogen | % | 0.31 | 0.26 | 0.25 | 0.33 | 0.21 | 0.3-0.6 |
| Ammonium-N | kg/ha | 5.5 | 3.2 | 5.9 | 5.5 | 7.2 | - |
| Nitrate-N | kg/ha | 91.2 | 68.1 | 60.9 | 103.9 | 153.5 | - |
| Mineral N | kg/ha | 96.7 | 71.3 | 66.8 | 109.4 | 160.7 | - |
| | | **12/10** | **12/10** | **12/10** | **12/10** | **12/10** | |
| Ammonium-N | kg/ha | 8.9 | 10.4 | 11.9 | 10.4 | 7.4 | - |
| Nitrate-N | kg/ha | 13.4 | 11.9 | 10.4 | 8.9 | 7.5 | - |
| Mineral N | kg/ha | 22.3 | 22.3 | 22.3 | 19.3 | 14.9 | - |

## 3.4 Seed, inoculation and sowing

'Stamina 5' lucerne was used for all treatments (Table 3.2). Peat slurry was prepared by mixing inoculant bacterial culture with sufficient water to wet it and form a uniform adhesive coating around the seed. ALOSCA ® granules impregnated with *Rhizobia* were mixed with bare seed at the recommended rate of 10.5 kg/ha in the drill at sowing. The coated seed contained *Ensifer meliloti*, a contact fungicide against *Pythium,* molybdenum and lime. At sowing, to prevent contamination of one treatment by the other, the following order was used;

bare seed, ALOSCA ® mix, coated seed, peat slurry mix. The hoppers were pressure cleaned with air after coated seed sowings to maintain sterility.

**Table 3.2 Thousand seed weights (TSW) and corresponding calculated seed rates per hectare and per plot for each seed treatment.**

| Treatment | TSW (g) | Seed rate (kg/ha) | Seed rate (g/plot) |
|---|---|---|---|
| **ALOSCA ® seed** | 2.14 | 10.5 | 30.9 |
| **Bare seed** | 2.14 | 10.5 | 30.9 |
| **Coated seed** | 3.24 | 16.0 | 50 |
| **Peat seed** | 2.14 | 10.5 | 30.9 |

## 3.5 Measurements

### 3.5.1 Gravimetric soil moisture

Gravimetric soil moisture content (Figure 3.1) was measured from the top 10 mm of soil on the day of each sowing. From each treatment, twenty 50 g samples were randomly collected and mixed homogenously to make a collective sample weighing up to 250 g of soil from each treatment. These were oven dried at 95 $^{o}$C for at least 48 hours to constant weight. Values ranged from 7-14% gravimetric soil moisture across sowing dates, being driest for the final two sowing dates (<10%).

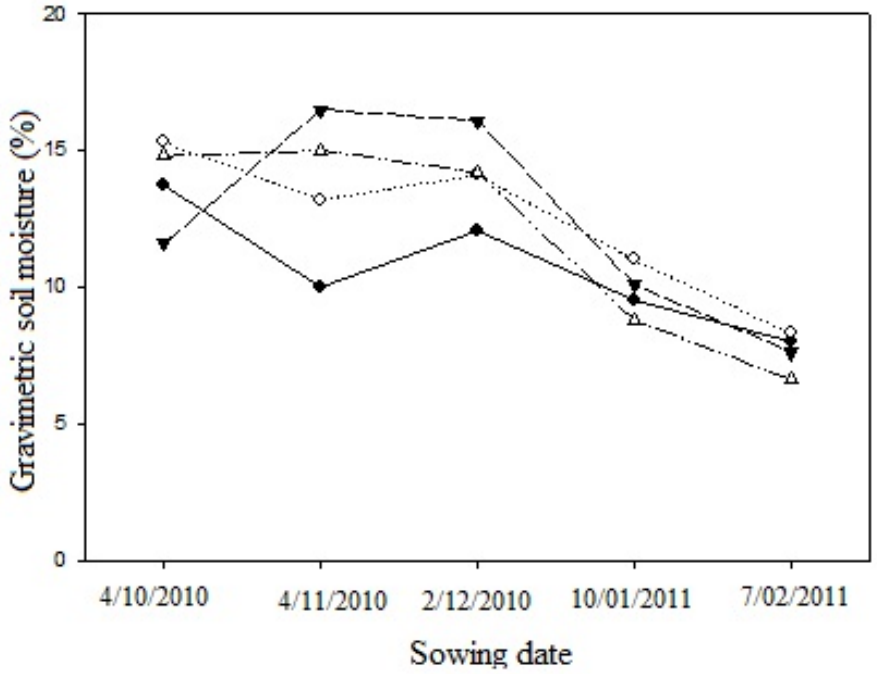


**Figure 3.1 Gravimetric soil moisture content of 'Stamina 5' seedling lucerne treated with a bare seed control (○), ALOSCA® (●), lime coating (▼) and peat (△) at establishment on five sowing dates at Lincoln University, Canterbury.**

### 3.5.2 Seedling emergence and initial plant population

The appearance of a spade leaf on seedlings was considered emergence. The number of emerged seedlings was determined from two 1 m long drill row sections from the middle 8 rows. These were fixed in each plot and observed every three days. Appearance of the 1st trifoliate leaf was also noted and counting ceased when plant numbers remained constant within the marked area for three consecutive measurements. The initial plant population was then derived from the final emergence counts.

### 3.5.3 Established population counts

The established lucerne population was determined from two 1 m long drill row sections from the middle 8 rows, 12 months after the first sowing date (4/10/2010). Soil surrounding the plants was excavated to 0.2 m depths to expose individual taproots for counts. The established plant population was then derived from the mean counts from each plot. A previous crown count 9 months after the initial sowing date was poorly correlated with taproot number and gave false establishment population figures hence the need to recount taproots 12 months after the initial sowing.

### 3.5.4 Canopy development and radiation interception

Canopy development was assessed from a combination of leaf appearance rate (LAR) and green area index (GAI).

#### *3.5.4.1 Leaf appearance rate (LAR)*

Leaf appearance was defined as the appearance of a node with a fully unfolded and visibly open trifoliate leaf in each plot. After emergence 5-10 stems were randomly selected and tagged for primary leaf counts every 3-5 days, until terminal bud appearance for each treatment. Only 'dominant main stems' were marked, namely the tallest one third of the shoot population, because these account for the majority (>80%) of the shoot yield in lucerne crops (Teixeira *et al.* 2007a).

#### *3.5.4.2 Green Area*

A destructive 10 plant sample (0.14 m) of random vigorously growing plants was taken every 10 days and analysed for green area between the 2 and 6 trifoliate leaf stage. Green area was determined by passing herbage through a Licor 3100 leaf area meter (Licor Inc, Lincoln, NE, USA).

#### *3.5.4.3 Radiation interception*

Fractional radiation interception was measured with a Sun Scan Canopy Analysis System (Delta-T Devices, Cambridge-England) at 10-14 day intervals, depending on the prevailing weather conditions (Plate 3.1). For this, 8-10 random below and above canopy measurements were taken per plot from the 8-10 trifoliate leaf stage until the end of the growth cycle. Radiation interception before canopy closure was validated through a paired sample analysed by both the Sun Scan Canopy Analysis System and the leaf area meter at the 8 trifoliate leaf stage. Daily radiation, to determine total photosynthetically active radiation (PAR) was obtained from Broadfield meteorological station, located 2 km North of the site.

**Plate 3.1 Sun Scan Canopy Analysis System (Delta-T Devices, Cambridge-England) used to measure fractional radiation interception at 10-14 day intervals.**

### 3.5.5 Reproductive development

The presence of visible buds and flowers were recorded at 7-10 day intervals on the same marked stems used for leaf appearance. Observations on marked stems were taken from seedling emergence until the onset of flowering. Reproductive development was recorded as the date when 50% of marked stems had visible buds (Teixeira *et al.* 2011). Thermal time requirements to reach the 50% buds visible ($Tt_0$-bv) stage were calculated (Section 2.13.1.1) and collectively with photoperiod (Pp; h), tested as predictors of $Tt_0$-bv.

### 3.5.6 Dry matter (DM)

Lucerne dry matter yields were randomly measured from 0.2 $m^2$ quadrats cut every 7-10 days from each plot and dried at 65 $^oC$ for at least 48 hours to constant weight.

### 3.5.7 Nodulation tests

A visible test was used for nodulation assessments. When 6 and 10 trifoliate leaves had unfolded, at flowering and 12 months after the initial sowing date, 20 plants were randomly excavated from each plot and nodulation scored visually on a 0-5 basis (Table 3.3). This accounted for nodule number, size, pigmentation and distribution as defined by Corbin *et al.* (1977).

**Table 3.3 Nodulation assesment scores (Corbin *et al.* 1977) used for quantifying nodule number and size based on distribution on the root.**

| | Distribution and number of effective nodules | |
|---|---|---|
| **Nodule score** | ***Crown***** | **Elsewhere** |
| **0** | 0 | 0 |
| **0.5** | 0 | 1-4 |
| **1** | 0 | 5-9 |
| **1.5** | 0 | ≥10 |
| **2** | Few | 0 |
| **2.5** | Few | Few |
| **3** | Many | Many |
| **4** | Many | Few |
| **5** | Many | 0 |

* Effectiveness judged on basis of nodule size and internal pigmentation; ineffective nodules not considered.
**crown regarded as top 50 mm of root system.

### 3.5.8 Meteorological conditions

Meteorological data were recorded from 1 October 2010 to 6 June 2011 when the bulk of the experimental measurements were taken. No data were available for established plant population counts and nodule scores observed outside the experimental period in October 2011. All meteorological data excluding soil and air temperature were taken from Broadfields Meteorological Station (NIWA, National Institute of Water and Atmospheric Research, New Zealand). Soil and air temperature were recorded on the experimental site using a HOBO U12 4-Channel External Data Logger (U12-008). For the soil temperature measurements, four sensors were buried 10 mm below the soil surface into random plots. The air temperature

sensors were positioned 1.2 m above the soil surface. The total rainfall and Penman potential evaporation (mm) from 1 October 2010 to 6 June 2011 were 367 mm and 888 mm respectively, giving a total deficit of 521 mm. The summary of meteorological data is shown in Figure 3.2. October, December, January and February were drier than the long term means whilst March and April received above long term average rainfall with up 34% more in April (Figure 3.2a). The annual mean temperature was 13.6 $^{o}$C varying from a monthly average of 10.3 $^{o}$C in June to 16.7 $^{o}$C in February. The lowest average daily total solar radiation was 5.3 MJ/m$^2$/day in May 2011 and the highest was 23.7 MJ/m$^2$/day in November 2010.

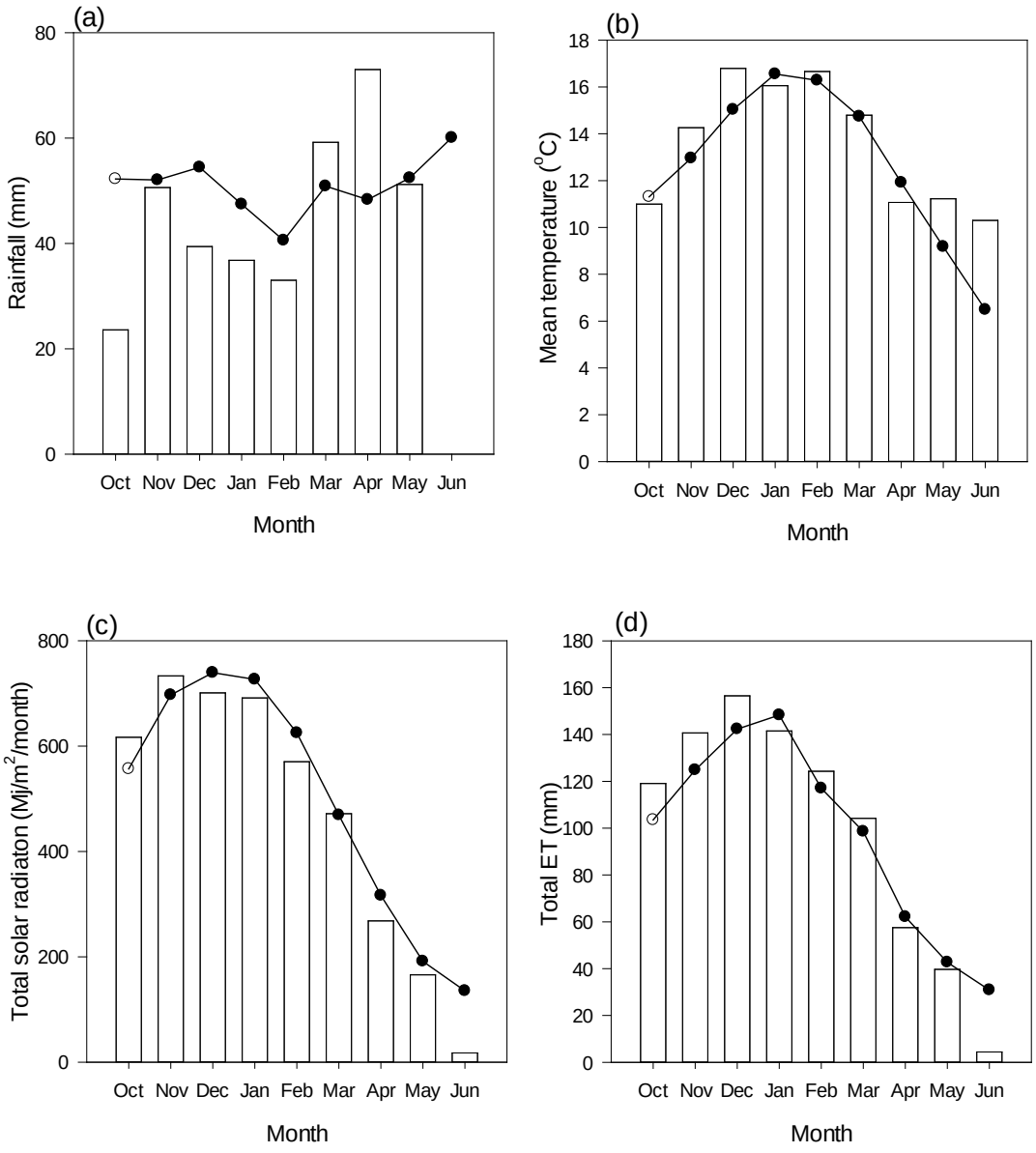


**Figure 3.2 Meteorological data from 1 October 2010 to 6 June 2011; long term mean (●) and experimental period (monthly) totals (☐) taken from Broadfields Meteorological station (NIWA, National Institute of Water and Atmospheric Research, New Zealand).**

### *3.5.8.1 Soil moisture content*

The lucerne experiment was rainfed (dryland) and soil moisture content was measured every 10-14 days by time domain reflectometry (TDR) at 0.2 m depths from the first sowing date until the end of June 2010. From these measurements temporal and spatial changes in the volumetric soil moisture content were observed for each plot and at each depth over time. Figure 3.3 shows the change in soil moisture content and rainfall over the experimental period from 0-0.2 m of the profile for each sowing date. In SD 1 the top 0.2 m of the soil had around 16% moisture at sowing. Rainfall was lower than the long term mean (LTM) in October (24 mm compared with 52 mm) and again from November to February (Figure 3.2a). Regardless of this, soil moisture content remained adequate between wilting point (9%) and field capacity (18%) for the first and second sowing dates, between October and December 2010. The 11 mm of rainfall, six days after the first sowing, on the 10th of October raised the soil moisture level above field capacity to 22%. It was maintained above field capacity by a series of intermittent rainfall events until after the third sowing in November 2010.

**Plate 3.2 Time domain reflectometry (TDR) for measuring soil moisture content at 0.2 m depths every 10-14 days.**

Volumetric soil moisture content declined in December from about 24% to 6% for crops from the first two sowing dates. The average monthly rainfall of 39 mm, was 28% lower than the LTM. This occurred at a time of active lucerne growth and canopy closure hence the high evapotranspiration rate. The 33 mm of rain in January raised the volumetric soil moisture to 9% in SD 1 and SD 2 and up to field capacity in SD3. This clearly indicates a higher moisture demand (transpiration rate) of plants as they grow and form a canopy than from bare soil. SD 4 was established in January after a fallow from September which gave a 30% volumetric moisture content. This declined to 25% during a dry spell in February when SD 5 was sown and continued to decline steadily until field capacity was returned at the end of March. In autumn, the soil moisture began to recharge after heavy rains, 14% and 34% above the LTM, in March and April, respectively. Throughout May and June, the soil moisture remained above 31% at a constant mean air temperature of 11 $^{o}$C.

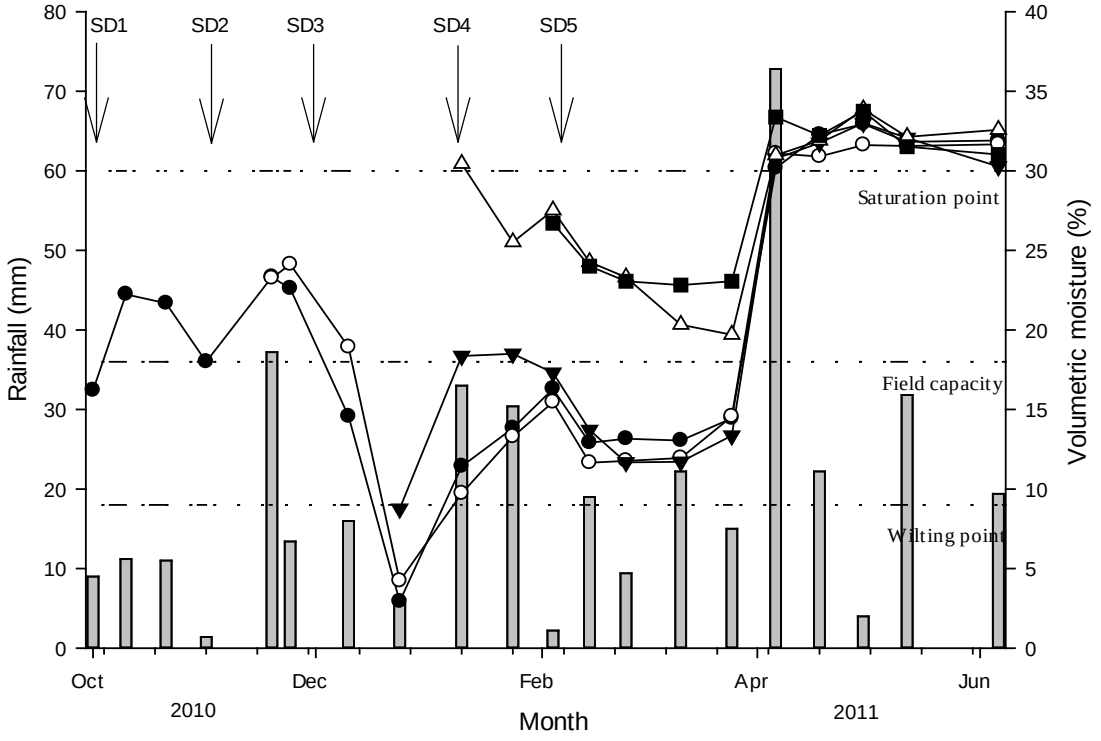


**Figure 3.3 Soil moisture content to 0.2 m depth and rainfall (mm) for 'Stamina 5'seedling lucerne crops established on five sowing dates (4/10/2010(●) , 4/11/2010(○), 2/12/2010(▼), 10/01/2011(△) and 7/02/2011(■)) at Lincoln University, Canterbury. Arrows represent actual sowing dates (SD).**

## 3.6 Calculations

### 3.6.1 Thermal time accumulation

Daily thermal time (Tt, $^{o}$Cd) was calculated as a sum of daily Tt using a broken stick threshold model (Fick *et al.* 1988; Robertson *et al.* 2002) where Tt is assumed to be zero for mean air temperatures ($T_{mean}$) below the base temperature ($T_b$) of 1.0 $^{o}$C (Jones & Kiniry 1986). Tt is accumulated linearly at a rate of 0.71 $^{o}$Cd/$^{o}$C from $T_b$ until 15 $^{o}$C and again at a rate of 1.0 $^{o}$Cd/$^{o}$C until the optimum temperature ($T_{opt}$) of 30 $^{o}$C (Teixeira *et al.* 2011), which was not exceeded throughout the study. An alternative threshold ($Tt_{b5}$) was used for leaf appearance calculations. This observed a linear increase in Tt from a base temperature of 5 $^{o}$C, a ($T_{opt}$) of 30 $^{o}$C followed by a linear decrease to a maximum (Tmax) of 40 $^{o}$C.

### 3.6.2 Leaf appearance rate

Leaf appearance ($^{o}$Cd/main stem node) was calculated as the linear regression between the number of primary leaves marked on the main stem and thermal time accumulation from emergence.

### 3.6.3 Leaf area expansion rate

Leaf area expansion rate (LAER, $m^2$ leaf/$m^2$ soil/ $^{o}$Cd) was calculated as the linear slope of the relationship between LAI and thermal time accumulation.

### 3.6.4 Nitrogen yield

Nitrogen concentration and yield were determined using Near Infra-red spectroscopy (NIR) calibration (Jones & Kiniry 1986). This was based on total N by Dumas combustion of milled (Cyclotec Sample Mill) dry lucerne samples. Total N yield was calculated using Equation 3:

**Total N yield (kg/ha) = %N herbage × kg DM/ha** **Equation 3**

### 3.6.5 Radiation use efficiency

The radiation use efficiency (RUE, g DM/MJ $PAR_i$) was derived from the linear slope between accumulated $PAR_i$ and total crop DM.

### 3.6.6 Photoperiod

Daily photoperiod (Pp) was derived by summation of photoperiod hours from sunrise to sunset on each day of sowing.

## 3.7 Statistical Analysis

Statistical analyses were performed using Genstat 12, release 12.1 (Lawes Agricultural Trust, Rothamsted experimental station, UK, 2009). Analysis of variance (ANOVA) was used to analyse all variates in a split plot experimental design. Fishers' protected least significant difference (LSD) test was used to determine the extent of variation between different levels of a factor when the ANOVA was significant ($\alpha$=0.05). The pooled standard error of the mean is used where mean data are reported.

# Chapter 4

# Results

Unless otherwise stated, there were no significant two way interactions between sowing date and seed treatment for any of the measured variables.

## 4.1 Emergence

Figure 4.1 shows the pattern of seedlings that emerged up to 30 days after sowing for each seed treatment and sowing date. Sowing dates 1, 2 and 3 took 30 days ($P<0.001$) to reach a stable initial plant population compared with 15 days for sowing date 4 and 10 days for sowing date 5. Coated seed consistently had the highest ($P<0.001$) initial plant population on all sowing dates. For example, in the first spring sowing (SD 1), 318 plants/$m^2$ or 64% of the seeds sown emerged compared with less than 200 plants/$m^2$ from the other seed treatments. A similar pattern of emergence was observed across all sowing dates with over 400 plants/$m^2$ from coated seed at initial emergence in SD 5, and about half that number ($P<0.001$) from the other treatments.

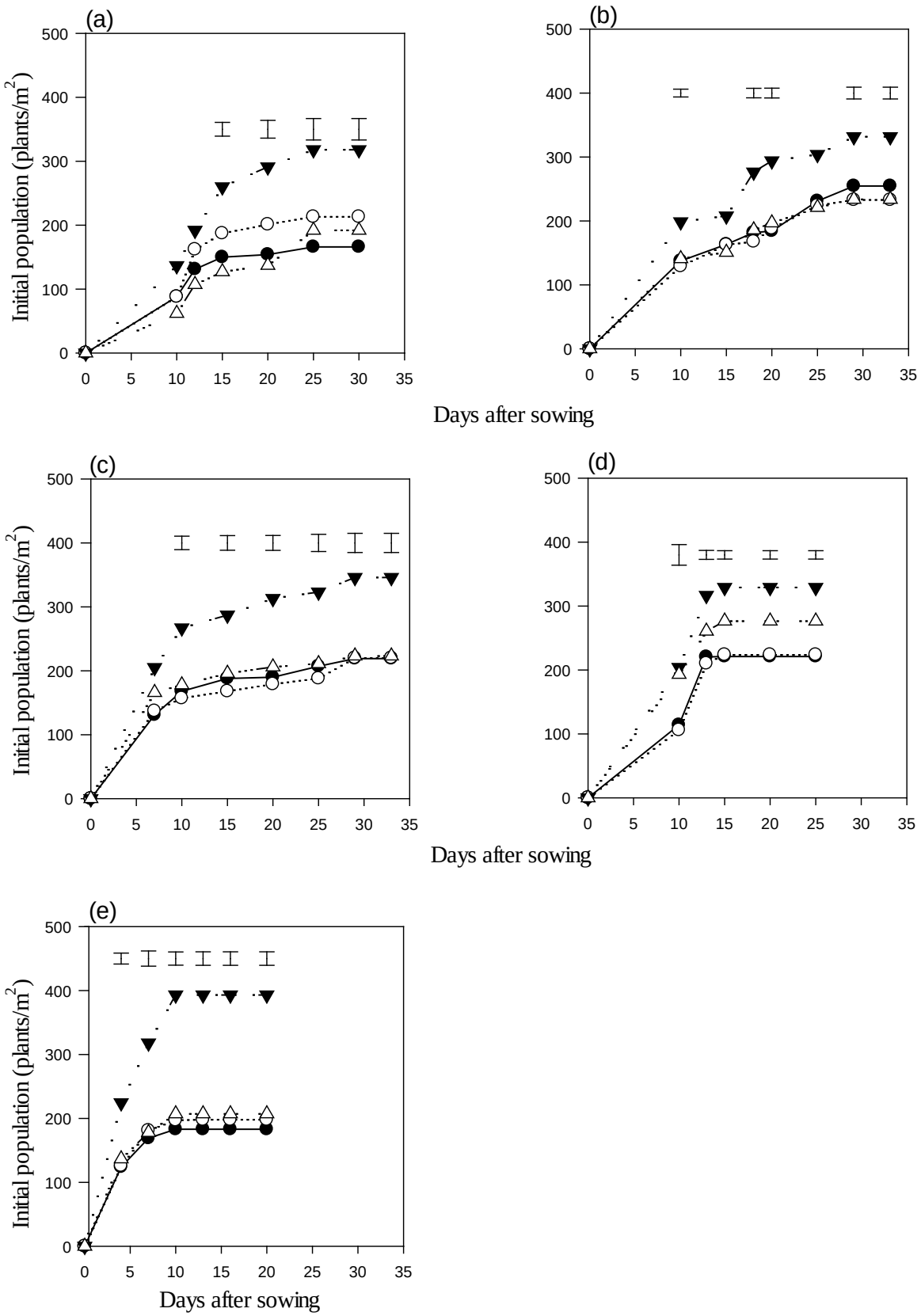


**Figure 4.1 Initial number of seedlings emerged after sowing on five dates (a-e; (4/10/2010), (4/11/2010), (2/12/2010), (10/01/2011), (7/02/2011)) and treated with a bare seed control (○), ALOSCA® (●), lime coating (▼) or peat inoculant (△) at Lincoln University, Canterbury in 2010. Error bars represent the standard error of means on each measurement day.**

SD 1 and 3 had the lowest (P<0.005) average thermal time requirement to emergence of 81 $^{o}$Cd whilst SD 2 and 4 required 159 $^{o}$Cd. Thermal time requirements to 75% emergence (Figure 4.1) were calculated using a broken stick function for thermal time accumulation against temperature (Teixeira *et al.* 2011). Logistic curves were then fitted to the emergence data and plotted against thermal time (Appendix 1). The fitted curves differed (P<0.001) among sowing dates but not seed treatments (P<0.64) (Table 4.1).

**Table 4.1 Thermal time to 75% ($Tt_b$=0$^{o}$C) of final emergence for 'Stamina 5' lucerne sown on five dates (a-e; (4/10/2010), (4/11/2010), (2/12/2010), (10/01/2011) (7/02/2011)) and treated with a bare seed control (BS), ALOSCA® (AS), lime coating (CS) or peat inoculant (PS) at Lincoln University Canterbury in 2010.**

| | Treatments | | | | |
|---|---|---|---|---|---|
| **Sowing date** | **BS** | **AS** | **CS** | **PS** | **Mean** |
| 4/10/2010 | 80 | 73 | 86 | 79 | 79 $_{de}$ |
| 4/11/20102 | 180 | 204 | 163 | 172 | 180 $_{a}$ |
| 2/12/2010 | 101 | 104 | 100 | 82 | 97 $_{cd}$ |
| 10/01/2011 | 141 | 141 | 135 | 135 | 138 $_{b}$ |
| 7/02/2011 | 64 | 64 | 90 | 64 | 70 $_{e}$ |
| Mean | 113 | 117 | 115 | 107 | 113 |
| F pr. 0.64 | SE. 6.04 | CV 23.9% | | | |

Means with letter subscripts in common are not significantly different ($\alpha = 0.05$, n = 45)

## 4.2 Establishment populations

Plant taproot counts, 12 months after the first sowing (October 2011) also showed differences (P<0.001) in plant populations amongst treatments but not among sowing dates. Coated seed maintained a higher (P<0.001) plant population with 250 plants/m$^2$ established compared with 191 plants/m$^2$ in the control and other treatments (Figure 4.2). There was a 27% decline in the plant population over winter for coated seed treatments from 344 to 250 plants/m$^2$ compared with an average of 13% for the other treatments.

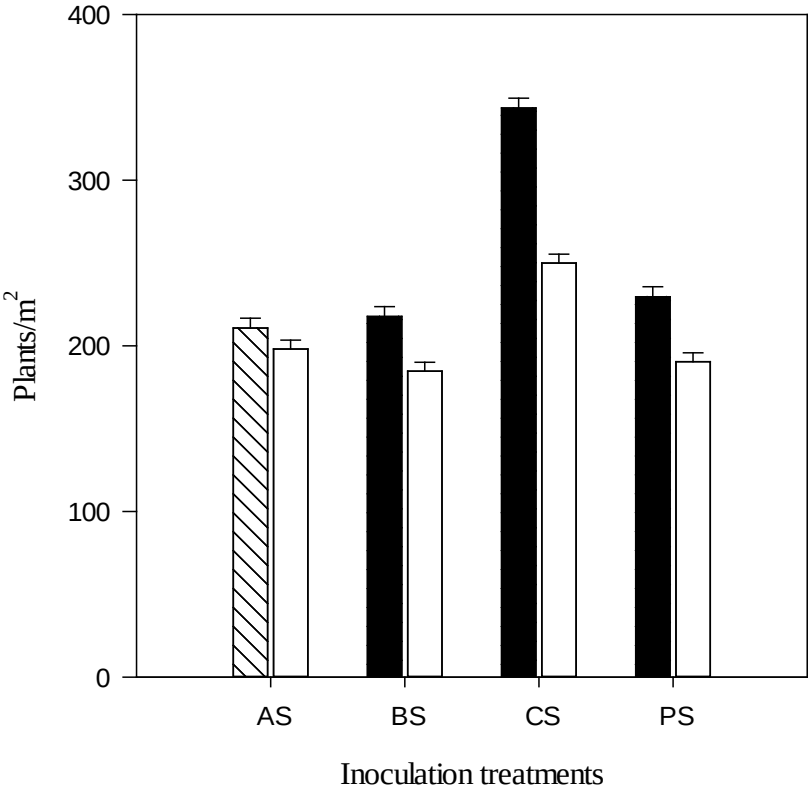


**Figure 4.2 Mean initial (■) and established (□) plant populations for 'Stamina 5' lucerne sown on five dates (a-e; (4/10/2010), (4/11/2010), (2/12/2010), (10/01/2011), (7/02/2011)) and treated with a bare seed control (BS), ALOSCA® (AS), lime coating (CS) or peat inoculant (PS) at Lincoln University, Canterbury in 2010. Error bars represent the standard error of means for each treatment.**

## 4.3 Vegetative development

### 4.3.1 Leaf appearance (thermal time)

The interval between the appearance of successive main stem leaves (phyllochron) was analysed for all treatments by regressing leaf appearance against thermal time for the linear phase in each sowing date (Figure 4.3). A base temperature of 5 $^{o}$C was used in this analysis (Section 3.6.1). In most cases the relationship between the number of nodes on the main stem was linear and unaffected by seed treatment. However, the slope of the regression differed among sowing dates and was less consistent ($R^2$<0.83) for SD 1 (Table 4.2). The phyllochron lengthened as the season progressed for all sowings dates. For SD 1 and SD 2, a bilinear pattern of leaf appearance was observed. The first three leaves took almost 400 $^{o}$Cd to be fully emerged and then there was a rapid increase in the rate of leaf appearance with a new leaf every 25 $^{o}$Cd. Averaged over the entire seedling duration, the mean phyllochron was 53 $^{o}$Cd.

Similarly for SD 2, the first three leaves took over 200 $^{o}$Cd to appear but averaged over the seedling growth period, the phyllochron was 46 $^{o}$Cd (Table 4.2). There was a more consistent linear node appearance for the later sowing dates but the phyllochron increased from 57 $^{o}$Cd for SD 3 to 65 $^{o}$Cd for SD 5.

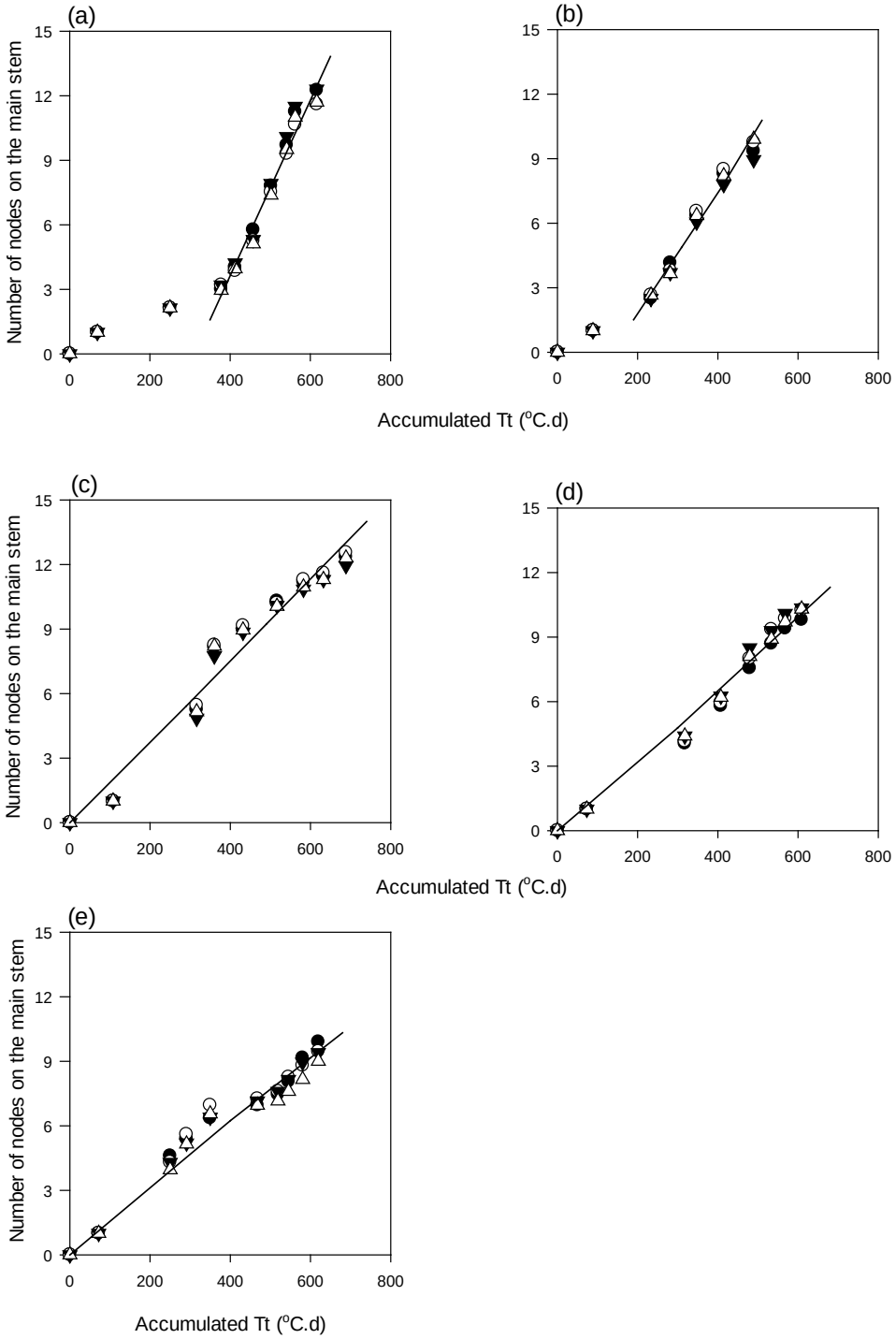


**Figure 4.3 'Stamina 5' lucerne node appearance against thermal time ($T_b$=0 $^{o}$C) after emergence on five sowing dates (a-e; (4/10/2010), (4/11/2010), (2/12/2010), (10/01/2011), (07/02/2011)) treated with a bare seed control (○), ALOSCA® (●), lime coating (▼) or peat incolant (△) at Lincoln University, Canterbury in 2010.**

**Table 4.2. The phyllochron of 'Stamina 5' seedling lucerne crops sown on five sowing dates (a-e; (4/10/2010), (4/11/2010), (2/12/2010), (10/01/2011), (07/02/2011)) treated with a bare seed control , ALOSCA®, lime coating (▼) or peat inoculant at Lincoln University, Canterbury in 2010.**

| | Sowing date | Leaf appearance rate | $R^2$ (%) | Phyllochron ($^oCd$) | Days/node |
|---|---|---|---|---|---|
| (1) | 4/10/2010 | 0.0408± 0.00086 | 83 | 44.6 | 4.6 |
| (2) | 4/11/20102 | 0.0281± 0.00086 | 93 | 45.8 | 4.0 |
| (3) | 2/12/2010 | 0.0165± 0.00066 | 98 | 51.8 | 4.1 |
| (4) | 10/01/2011 | 0.0191± 0.00061 | 97 | 57.1 | 5.5 |
| (5) | 7/02/2011 | 0.0115± 0.00038 | 95 | 64.9 | 7.4 |

## 4.4 Reproductive development

### 4.4.1 Time to reach 50% buds visible

Only three sowing dates; SD 1, SD 2 and SD 3 were included in the analysis of reproductive development because they were the only seedling crops that flowered. The number of days from emergence to 50% bud visible differed (P<0.001) across sowing dates but not amongst seed treatments. It took 65 days to flowering from the first sowing, 52 days from the second, and 59 days from the third. The thermal time required to reach 50% bud visible ($Tt_0$-bv) also differed (P<0.001) and ranged from 686 $^oCd$ in SD 1 to a projected minimum of 651 $^oCd$ in SD 2 before rapidly ascending to 761 $^oCd$ in SD 3 (Table 4.3).

**Table 4.3 Thermal time ($Tt_b$ =0$^o$C) requirement for 50% appearance of buds ($Tt_0$-bv) for 'Stamina 5' lucerne sown on three dates ;(4/10/2010), (4/11/2010), (2/12/2010) and treated with a bare seed control (BS), ALOSCA® (AS), lime coating (CS) or peat inoculant (PS) at Lincoln University, Canterbury 2010.**

| | Sowing date | BS | AS | CS | PS | Means |
|---|---|---|---|---|---|---|
| (1) | 4/10/2010 | 685 | 688 | 680 | 692 | $686_b$ |
| (2) | 4/11/20102 | 654 | 644 | 648 | 657 | $651_c$ |
| (3) | 2/12/2010 | 759 | 753 | 773 | 757 | $761_a$ |
| | Mean | 699 | 695 | 700 | 702 | 699 |
| | F pr.0. 63 | SE. 5.68 | CV. 1.9 | | | |

Means with letter subscripts in common are not significantly different ($\alpha = 0.05$, n = 27)

## 4.5 Dry matter yield

### 4.5.1 Total seedling dry matter yield

Accumulated crop dry matter (DM) yields over the measurement period differed amongst sowing dates but not seed treatments. DM yields decreased (P<0.001) with each sowing as the season progressed (Figure 4.4). SD 1 accumulated 15 t/ha which was (P<0.001) more than the 4.0 t/ha from SD 4. SD 1 grew for up to 200 days after emergence compared with a single defoliation after 100 days for SD 5.

## 4.6 Nitrogen yield

Plant nitrogen yields for the first defoliation varied amongst sowing dates. SD 3 had the highest (P<0.001) N yield of 128.7 kg/ha which was higher (P<0.001) than 66.4 kg/ha in SD 5 (Table 4.4). No differences in N yield were observed amongst seed treatments.

**Table 4.4 Nitrogen yield (kg/ha) at first defoliation for 'Stamina 5' seedling lucerne sowing on five dates (4/10/2010), (4/11/2010), (2/12/2010), (10/01/2011) (7/02/2011) and treated with a bare seed control (BS), ALOSCA® (AS), lime coating (CS) or peat inoculant (PS) at Lincoln University, Canterbury.**

| | | Treatments | | | | |
|---|---|---|---|---|---|---|
| | **Sowing date** | **BS** | **AS** | **CS** | **PS** | **Mean** |
| (1) | 4/10/2010 | 73.7 | 88.5 | 74.2 | 80.4 | $79.2_{bcd}$ |
| (2) | 4/11/2010 | 82.2 | 92.6 | 73.3 | 85.7 | $83.4_{bc}$ |
| (3) | 2/12/2010 | 137.1 | 118.2 | 124.2 | 135.4 | $128.7_{a}$ |
| (4) | 10/01/2011 | 84.3 | 94.0 | 77.5 | 85.3 | $85.3_{b}$ |
| (5) | 7/02/2011 | 64.5 | 69.6 | 73.5 | 58.1 | $66.4_{d}$ |
| | Mean | 88.4 | 92.6 | 84.5 | 88.9 | 88.6 |
| | F pr. 0.46 | SE. 3.5 | CV 17.8% | | | |

Means with letter subscripts in common are not significantly different ($\alpha = 0.05$, n = 45)

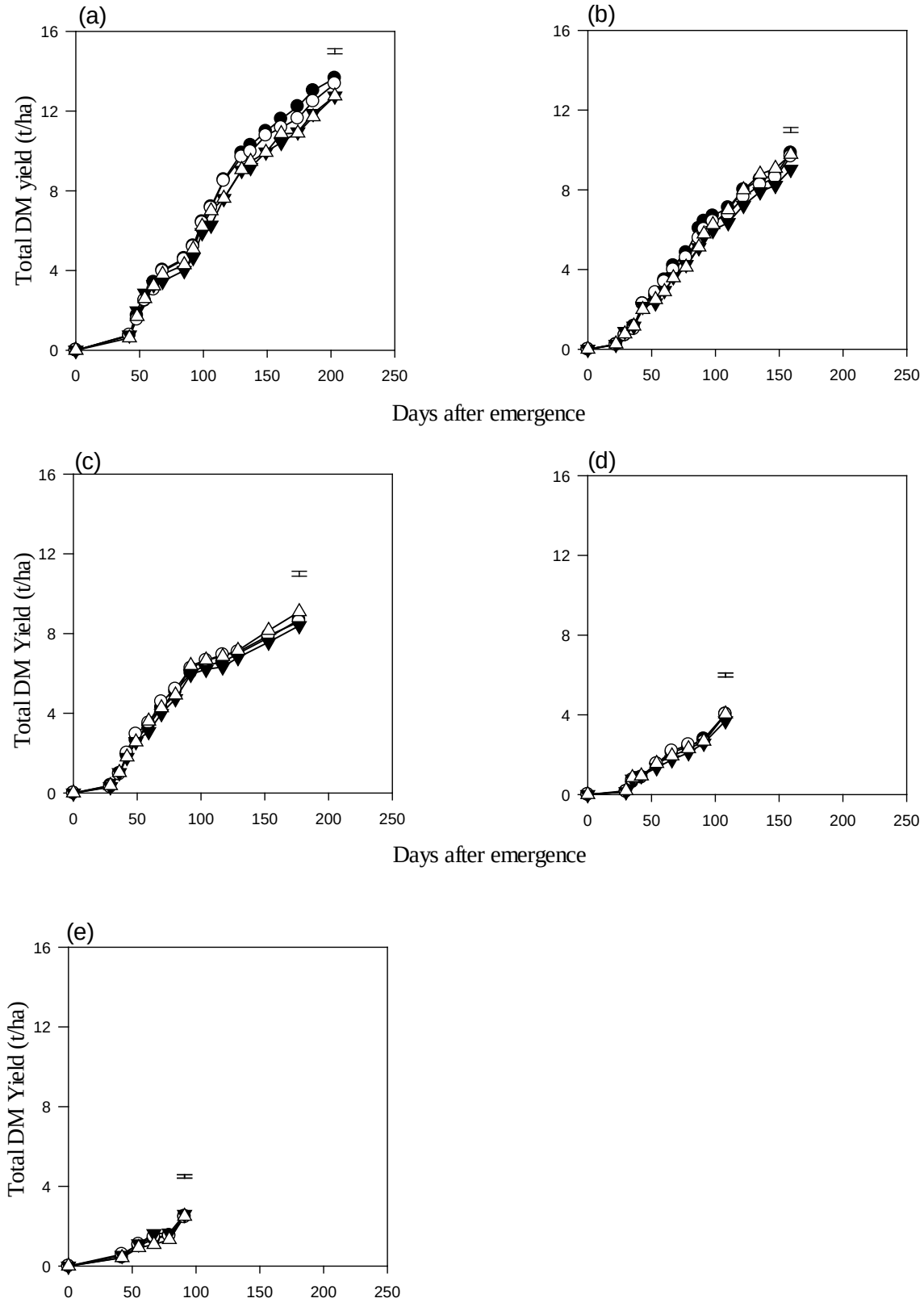


**Figure 4.4 'Stamina 5' lucerne dry matter (DM) accumulation over the growing season from sowing on five dates (a-e; (4/10/2010), (4/11/2010), (2/12/2010), (7/02/2011), (10/01/2011)) and treated with a bare seed control (○), ALOSCA® (●), lime coating (▼) or peat incolant (△) at Lincoln University, Canterbury. Error bars represent the standard error of means.**

Root nodule score at flowering and 12 months after the first sowing (October 2011) showed differences (P<0.001) amongst sowing dates but not among treatments. SD 2 maintained a consistently higher (P<0.001) mean nodulation score of 2.3 compared with 0.6 in SD 5 (Figure 4.5). Generally, SD 1 up to SD 3 had higher (P<0.001) nodulation scores than SD 4 and SD 5 in both observations.

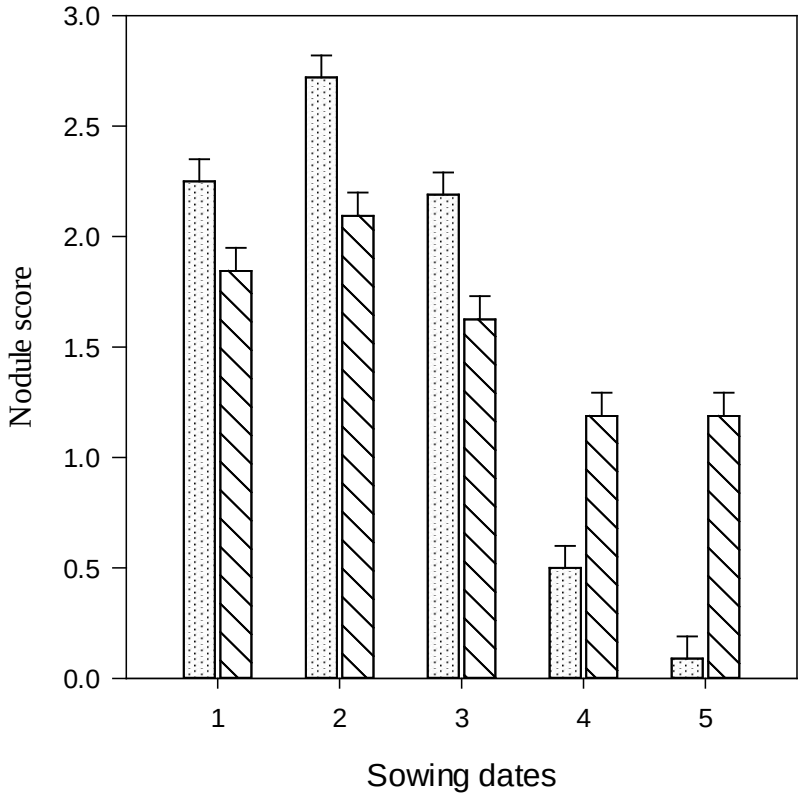


**Figure 4.5 Nodulation score at flowering ( ) and 12 months after the first sowing ( ) of 'Stamina 5' lucerne sown on five dates (1-5; (4/10/2010), (4/11/2010), (2/12/2010), (10/01/2011), (7/02/2011)) and treated with a bare seed control, ALOSCA®, lime coating or peat inoculant at Lincoln University, Canterbury. Error bars represent the standard error of means for each treatment.**

Leaf and stem metabolisable energy (ME) and nitrogen concentration differed (P<0.001) amongst sowing dates but not seed treatments. ME in the leaf ranged from 11.1 in SD 1 to 11.5 in SD 3 and nitrogen concentration increased with sowing date from 3.1% in SD 1 to 4.9% in SD 5 (Figure 4.6). In all sowing dates, leaf ME and N concentration were higher than those found in the stem.

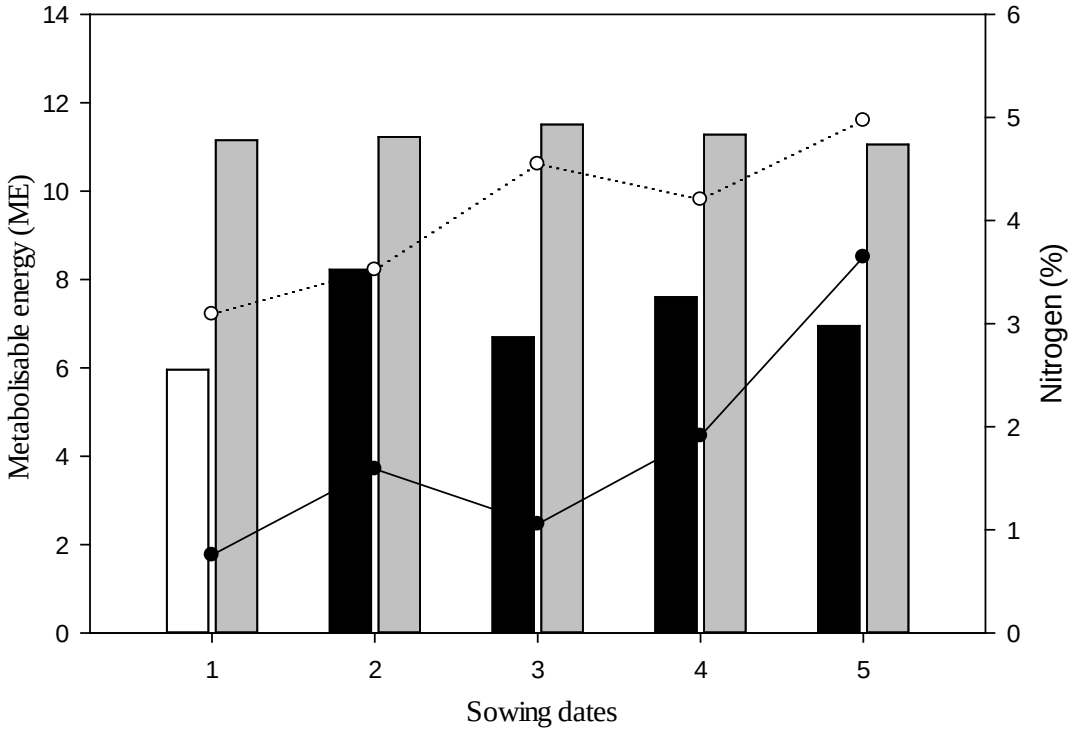


**Figure 4.6 Stem (■) and leaf (▒) metabolisable energy (ME) and stem (●) and leaf (○) nitrogen concentration of 'Stamina 5' seedling lucerne sown on five dates (1-5; 4/10/2010), (4/11/2010), (2/12/2010), (10/01/2011) (7/02/2011) at Lincoln University, Canterbury.**

## 4.7 Seedling and regrowth dry matter yield

In Figure 4.7 the number of defoliations and individual regrowth cycles are illustrated. The seedling crop in SD 1 yielded 60% more (P<0.001) DM than that in SD 5 and was defoliated after 1100 $^{o}$Cd compared with 1300 $^{o}$Cd for SD 5.

Linear regressions of the initial seedling crop growth and then individual regrowth crop dry matter against thermal time were used to analyse the dry matter production per °Cd. A base temperature of 1 °C was used in this analysis (Moot *et al.* 2000; Teixeira *et al.* 2011). There was a difference (P<0.001) in DM production per °Cd between sowing dates but no difference between seed treatment for seedling crops. SD1 had the highest DM production of 3.86±0.045 kg DM/ °Cd and SD 5 had the lowest DM production of 1.60±0.085 kg DM/ °Cd. There was also a difference (P<0.001) in dry matter production per °Cd between sowing dates for the regrowth crops. The first regrowth crop of SD 1 had the highest dry matter production of 1.83±0.18 kg DM/ °Cd compared with 0.03±0.19 kg DM/ °Cd for SD 4.

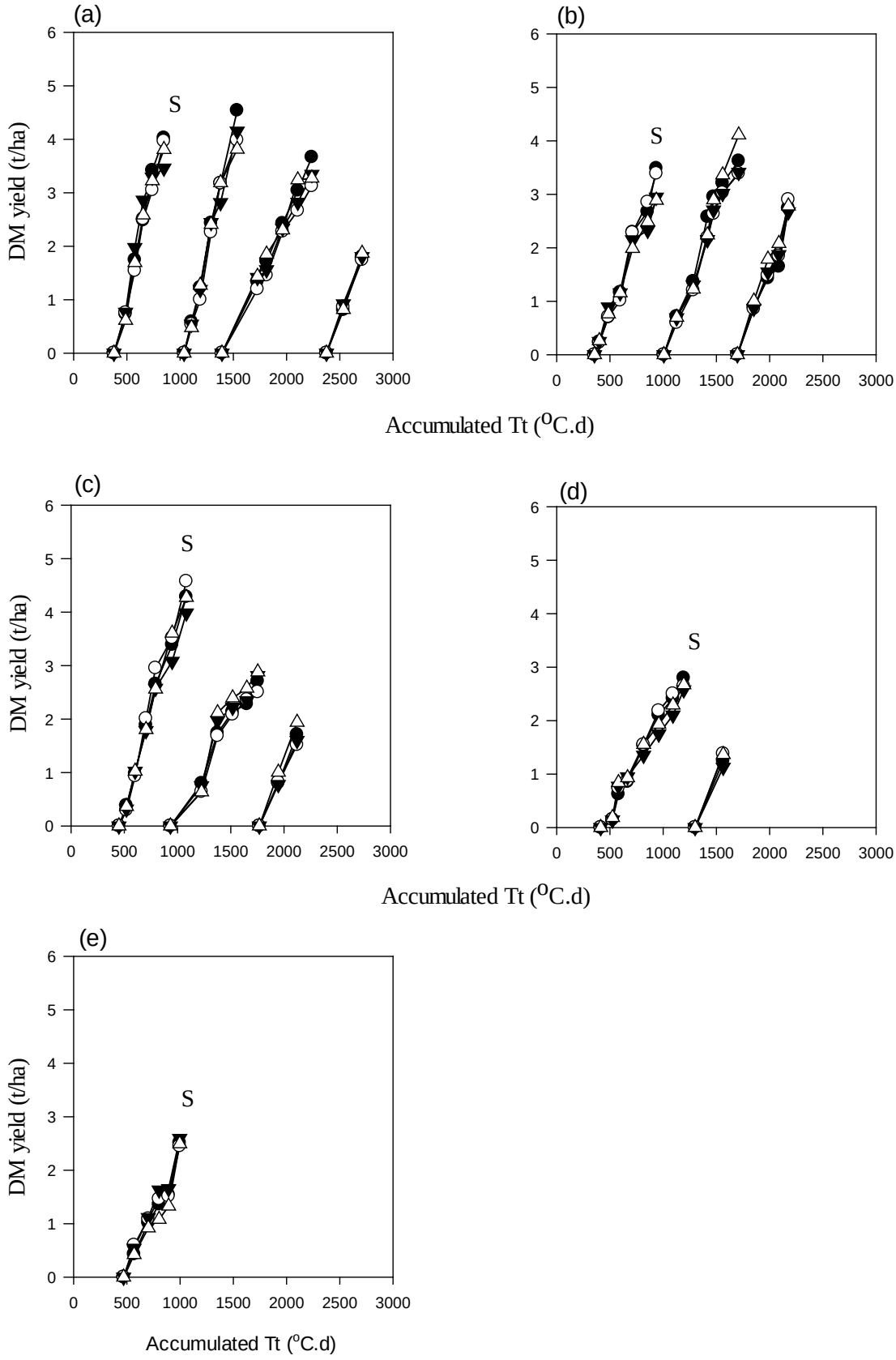


**Figure 4.7 Dry matter (DM) yield of seedling (S) and regrowth 'Stamina 5' lucerne against thermal time (Tb=1°C) for crops sown on five dates dates (a-e; (4/10/2010), (4/11/2010), (2/12/2010), (10/01/2011), (7/02/2011)) and treated with a bare seed control (○), ALOSCA® (●), lime coating (▼) or peat inoculum (△) at Lincoln University, Canterbury in 2010.**

## 4.8 Light interception

Neither sowing date nor inoculation treatment affected leaf area index (LAI) throughout the growing period. Regrowth crops for all sowing dates attained the highest LAI of 4 earlier ($P<0.001$) than seedling crops as observed by the steeper slope of the regression line for seedling crops (Figure 4.8). Seedling crops had a lower ($P<0.001$) leaf area expansion rate (LAER) of 0.006±0.0009 LAI/°Cd compared with 0.0075±0.0011 LAI/°Cd in regrowth crops.

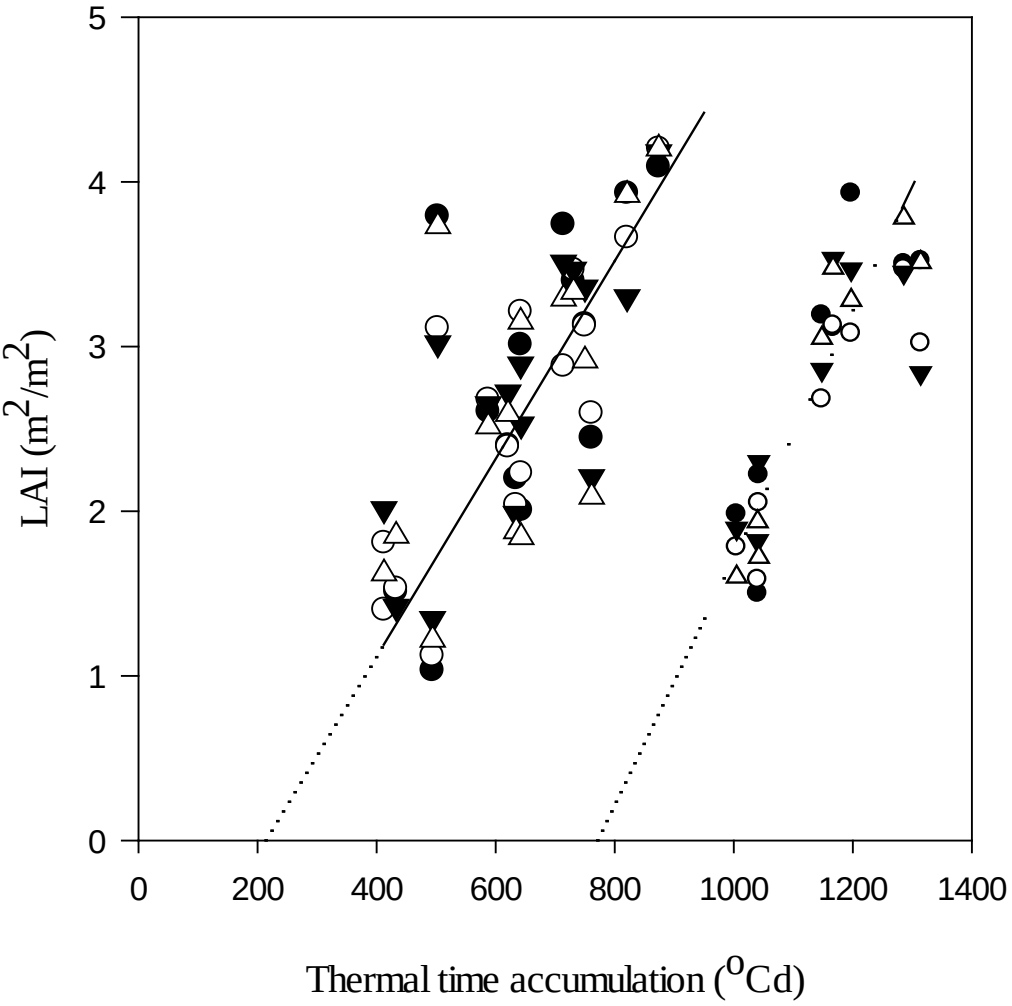


**Figure 4.8 Leaf area index against thermal time accumulation ($Tt_b$=0°C) after emergence for seedling (dashed regression line) (y=0.0075x-5.78; $R^2$ = 76%) and regrowth (solid regression line) (y=0.006x-1.282; $R^2$=74%) 'Stamina 5' lucerne crops sown on five dates dates (a-e; (4/10/2010), (4/11/2010), (2/12/2010), (10/01/2011), (7/02/2011)) and treated with a bare seed control (○), ALOSCA® (●), lime coating (▼) or peat inoculum (△) at Lincoln University, Canterbury. Dotted lines were extrapolated to the x-axis.**

Seedling and regrowth crops showed a similar pattern of increasing fractional light interception with leaf area index (Figure 4.9). All crops from the five sowing dates achieved 95% light interception at a $LAI_{crit}$ of 4.0. Consequently, the calculated extinction coefficient for incoming PAR (k) was 0.75.

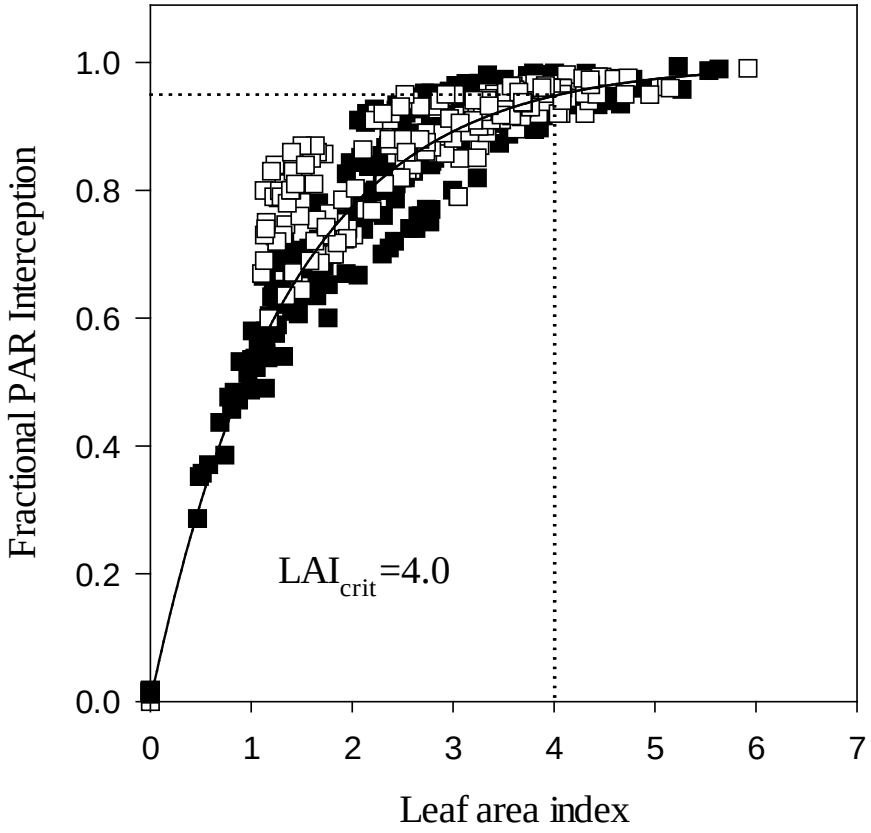


**Figure 4.9. Fractional interception of photosynethically active radiation against leaf area index for 'Stamina 5' lucerne at seedling (■) and regrowth stages (□) grown at Lincoln University, Canterbury in 2010. The equation for the relationship was; y=1-e-0.75x; R2=0.96.**

## 4.9 Radiation use efficiency for shoot production

The RUE differed amongst sowing dates and declined consistently as the season progressed from spring to autumn. SD 1 had a RUE of 3.9 kg/ $^{o}$Cd which was 59% higher than 1.6 kg/ $^{o}$Cd for SD 5. Seed treatment had no effect on RUE but seedling and regrowth crops showed a linear increase in shoot biomass against accumulated $PAR_i$. The highest individual crop yields for both crop cycles were ~450 g DM/$m^2$ or 4.5 t DM/ha (Figure 4.10). To achieve this, seedling crops required the interception of ~450 MJ $PAR_i/m^2$ compared with ~300 MJ

$PAR_i/m^2$ for regrowth crops. Therefore $RUE_{shoot}$ for seedling crops was 0.90±0.033 g or 18% lower (P<0.001) than the 1.11±0.065 g DM MJ $PAR_i/ m^2$ for regrowth crops.

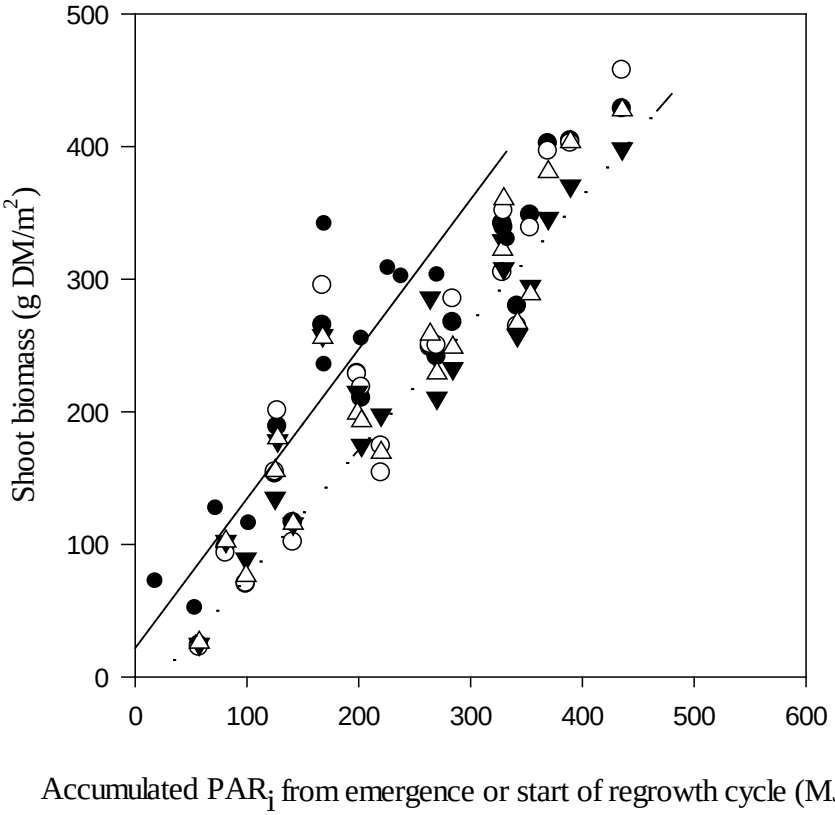


**Figure 4.10 Shoot biomass against intercepted photosynthetically active radiation ($PAR_i$) accumulated after emergence of 'Stamina 5' lucerne seedlings (dashed regression line) or regrowth crops (solid regression line) sown on five dates (a-e; (4/10/2010), (4/11/2010), (2/12/2010), (10/01/2011), (7/02/2011)) and treated with a bare seed control (○), ALOSCA® (●), lime coating (▼) or peat inoculum (△) at Lincoln University, Canterbury. The regression coefficients were y=0.904x-20.614; $R^2$=0.90 for seedlings and y=1.112x+21.849; $R^2$=0.89 for regrowth crops.**

# Chapter 5
# Agronomic Discussion

## 5.1 Emergence

Inoculation of 'Stamina 5' lucerne with either peat slurry or ALOSCA® was found to have no benefit on emergence or the final plant population compared with the bare seed control. However, coated seed produced a consistently higher ($P<0.001$) initial plant population in all five sowing dates compared with the other seed treatments (Figure 4.2). The average final emergence of 331 plants/$m^2$ was 68% of the 490 seeds/$m^2$ sown. The higher emergence exhibited by the coated seed treatment may be attributed to its ballistic seed which weighs approximately 1.6 times more than uncoated seed thereby enabling better ground penetration and increased seed to soil contact. In addition, the seed coating consisted of lime, molybdenum and a contact fungicide effective against *Pythium*. This combination may have improved the soil environment around the seed making it favourable for optimum seedling establishment. Despite having relatively lower emergence rates than coated seed, ALOSCA®, peat treated seed and the bare seed control still had an adequate plant stand. This confirms work by Palmer & Wynn-Williams (1976) who reported that maximum yield was attained at plant populations as low as 30 plants/$m^2$. They suggested that, provided the seeding rate is sufficient to give 30 plants/$m^2$ in the season following sowing, then stand life became independent of the initial seeding rate (Section 2.11).

Emergence rates were slower for SD 1 than other sowing dates (Figure 4.1). Only 93 plants/$m^2$ emerged 10 DAS compared with 166 plants/$m^2$ in SD 2, 3 and 4 and 185 plants/$m^2$ in SD 5. Both gravimetric (14%) and volumetric (16%) soil moisture content at the time of sowing were adequate for germination. Therefore, the slower ($P<0.005$) initial rate of emergence and time to the third trifoliate leaf may have been the result of the low soil temperatures (12 $^oC$) prevalent during late spring. SD 2 showed an inconsistent pattern of emergence. It took 30 days to reach final emergence compared with 25 days in SD 1, 15 days in SD 4 and 10 days in SD 5. The gravimetric soil moisture content of 14% for SD 2 was adequate for germination and emergence. However around 10 DAS soil temperatures increased to near optimum (18 $^oC$), and soil moisture was quickly evaporated which caused emergence to level off. This was particularly in the fast growing coated seed treatment (Figure

4.1b). Rainfall of 37 mm (Figure 3.3) 20 days after the second sowing appears to have restarted emergence as the population then increased again up to 30 DAS. This two stage emergence, due to dry soil conditions, has been noted commercially on farm with some indication that the levelling off was greater for the coated seed than other treatments. The gravimetric soil surface moisture content of 7.5% for SD 5 (Figure 3.1) indicates those seeds were sown into the driest conditions. Despite this, the emergence rate was rapid and the initial population achieved over just 10 days. This highlights the importance of a summer fallow for establishment. The volumetric soil moisture content for SD 5 was still 28% at sowing compared with 15% for the crops sown in spring. The conservation of moisture in the soil profile for SD 5 appears to have been sufficient to enable germinating seedlings to survive. At this time the soil temperature averaged 20 $^{o}$C which would aid rapid germination and emergence. Of note was the higher initial population from the coated seed treatment, particularly when the soil surface was dry. This contradicts the expectation that ALOSCA® would have superior performance under such conditions (Section 2.6.3). The initial emergence populations were over 40% of seeds sown regardless of conditions at sowing. This level of establishment was consistent with previous reports for lucerne (Wynn-Williams 1982).

Assuming soil moisture and temperature are non limiting, emergence can be expected after the same amount of thermal time regardless of sowing date. Deviation from this value suggests a limitation at emergence. Thermal time accumulation for emergence from SD 1, 3 and 5 was consistently 80 °Cd (Table 4.1), despite the range in soil temperatures from 11-16.8 $^{o}$C (Figure 3.2b). This value was slightly lower than the 99 °Cd observed by Fletcher (2000) for ‘Kaituna’ lucerne. In contrast, SD 2 had the highest ($P<0.001$) thermal time requirements of 181 °Cd. This delay in emergence was likely to be the result of the low gravimetric soil moisture content of 13.7% (Figure 3.1) in the top 10 mm of the soil at the time of sowing. As discussed, this caused a two step germination process. This was followed by a dry period until rainfall in early December. Despite differences in the patterns of emergence, all sowing dates achieved successful initial emergence of lucerne crops despite some delays when gravimetric moisture content was less than 10%. For SD 4 the delay was short because of the 21.4 mm of rainfall 8 days after sowing (Figure 3.3).

## 5.2 Established populations

Established populations were recounted in October 2011. These remained higher ($P<0.001$) for coated seed than all other treatments including the bare seed control. All populations were however above the minimum stand requirement of 30 plants/$m^2$ suggested by Palmer & Wynn-Williams (1976) or the 43 plants/$m^2$ Teixeira *et al.* (2007a) considered necessary to obtain maximum yields. Established populations were lower than emerged populations in all treatments, particularly in the coated seed which had a 27% decline from 344 to 250 plants/$m^2$ (Section 4.2). This highlights the occurrence of self thinning through plant death during autumn and winter growth periods. Thies *et al.* (2001) (Section 2.11) and Teixeira *et al.* (2007a) reported a 40 to 50% decline in plant population after the first year and second year respectively, a value almost double that reported in this study. The higher reduction in population noted in the initially overcrowded coated seed can be attributed to an exponential decay in plant population due to the mortality of smaller more suppressed plants failing to reach the upper canopy strata to capture sufficient light and maintain a positive carbon balance (Sackville Hamilton *et al.* 1995). Sims (1975) reported that stands with higher emergence populations suffered the highest plant mortalities during growth and ultimately established a final plant population of only 45.7% of seed sown due to competition and self thinning. Thus, the initial success of coated seed at establishment, may not lead to superior lucerne crop yields.

## 5.3 Dry matter

The highest accumulated DM yield of 15 t/ha from the earliest October sowing was comparable with previous experimental results for this location. Mills *et al.* (2008) reported annual DM yields of 13.1-18.5 t/ha on dryland lucerne over four years. SD 1 had four defoliations and yielded 33% more DM than SD 2 and 3 which both were defoliated three times throughout the growing season. This was expected because of the longer period of growth and opportunity for light interception compared with the later sowing dates. Consistent with this study, Frame *et al.* (1998) documented higher lucerne yields in spring rotations compared with lower ones in summer and autumn regrowth cycles. Lucerne DM yields decreased ($P<0.001$) with each sowing as the season progressed from spring to autumn.

SD 4 and 5 grown in autumn under favourable moisture conditions above field capacity (18%), had the lowest ($P<0.001$) cumulative DM yields of 4 and 2.5 t/ha. This was an

expected outcome at this time of the year and was previously ascribed to a direct response to short photoperiods and low temperatures (hence the fewer defoliations) (Teixeira *et al.* 2008) and consequent changes in source/sink relationships (Teixeira 2006) leading to preferential allocation of assimilates below ground. This is consistent with Noquet *et al.* (2001) who showed a 30% greater partitioning of nitrogen to lucerne roots under an 8 h photoperiod than under a 16 h photoperiod. Also, Brown *et al.* (2006) observed that biomass partitioning to shoots ($p_{shoot}$) decreased from 0.90 in early spring to 0.67 in spring and summer and then decreased to 0.35 in early autumn. This was consistent with the low RUE of 1.6 kg/ °Cd noted for SD 5 compared with 3.9 kg/ °Cd for SD 1 in this study. The 3.9 kg/ °Cd recorded for SD 1 is comparable to 4 kg/ °Cd reported by Mills *et al.* (2008) in the spring of 2002 for lucerne on the same site. The low volumetric soil moisture content of 12% experienced between February and March appears to have affected the rate of DM accumulation in SD 3, 100 DAE. A rainfall event of 73 mm in April resulted in a rapid recovery and a total yield of 9 t/ha for the growing season.

## 5.4 Nodulation and N concentration

Amongst the four growth stages proposed for nodule assessment (Section 3.5.7), flowering and 12 months after the first sowing date (Figure 4.5) were the only two that showed active nodulation. Previous nodule assessments done by Blair (1971) reported 30% or less plants nodulated 6-8 weeks after sowing. This was attributed to acidic soil conditions at the time of sowing. In the present study however, soil pH ranged from 5.5-6 (Table 3.1) in all sowing dates thus eliminating the possibility of unfavourable soil conditions including Al toxicity (Section 2.7.1) as possible causes of poor nodulation. According to Vance *et al.* (1988) large quantities of inorganic N in the rhizosphere inhibit N fixation. The poor nodulation observed at the 6 and 10 trifoliate leaf stages can thus be attributed to the exceptionally high soil mineral N concentration recorded at each sowing (Table 3.1). A general trend of increased nodulation is apparent when the soil mineral N content is low and vice versa (Figure 4.5). Results from this study contradict reports by Horikawa and Ohtsuka (1996) that lime coated lucerne seed experiences a rapid degree of nodulation and early seedling growth compared with peat inoculated seed. All seed treatments yielded alike including the bare seed control and no differences were observed in nodule score amongst them. This confirms the likelihood of a resident indigenous rhizobial strain in the experimental site.

Leaf ME values for all sowing dates remained consistently above 10.5 regardless of variation in N concentrations. This indicates that the feed value of the seedling crops was of high quality at the end of the establishment cycle. SD 1 contained 97 kg N/ha at sowing and yielded 79 kg N/ha. This suggests that soil N was adequate to meet crop demand for this sowing date. A similar result was obtained for SD 2 with mineralisable soil N of 71 kg/ha at sowing with a yield of 83 kg N/ha for the seedling crop. Surprisingly for SD 3 only 67 kg N/ha was available as mineralisable N but the crop yielded 129 kg N. The deficit of 62 kg N was probably met from nitrogen fixation. Lucerne has the capacity to fix about 28 kg N/t DM produced. The high nodule score for SD1 to SD 3 at flowering suggests these crops had commenced N fixation to meet current or future N demand. In contrast, for SD 4 to SD 5 low nodule scores were apparent supporting the idea that the 135 kg N available from mineralisation was sufficient to meet the crop demand of 71 kg/ha. In year 2 the nodule scores of these crops had increased significantly which suggests mineral N supply was inadequate to meet crop demand.

## 5.5 Leaf appearance rate

Leaf appearance ranged from a maximum rate of four days per leaf (0.247 leaves per day) in SD 2 and SD 3 to 7.4 days per leaf (0.134 leaves per day) in SD 5. SD 3 (02/12/2011) had a leaf appearance rate of 0.187 (4.1 days per leaf) which was faster than 0.18 leaves per day (5.5 days per leaf) recalculated by Teixeira *et al.* (2011) for a seedling crop sown on the 5$^{th}$ of December. This leaf appearance rate of 5.5 days per leaf was however recorded in SD 4 (10/01/2011) of the present study. Published leaf appearance rates of 0.018 leaves per day (55 days per leaf) from Teixeira *et al.* (2011) are unrealistic and result from a decimal error in their published result (Teixeira *et al.* 2011). The rate of leaf appearance declined as the season progressed from SD 2 to SD 5. SD 1 did not however fit the pattern probably due to moisture stress or increased allocation of biomass underground.

According to Brown *et al.* (2003), lucerne growth occurs in two phases. An initial lag period characterized by slow growth and production of the first leaves, followed by a linear development period of increased light interception and rapid growth. The second trifoliate leaf appeared 40 DAS (360 $^{o}$Cd) in SD 1 compared with 20 DAS (232 $^{o}$Cd) in all other sowing dates. This lag phase of delayed growth in SD 1, despite favourable prevailing temperatures can be explained by moisture stress early in the season which probably affected leaf

expansion but not the plastochron or leaf development (Section 2.13.1). Although the volumetric soil moisture content did not go below field capacity between 0 and 40 DAS, rainfall readings for the months of October and November were relatively low (Figure 3.3). It is therefore possible for the uppermost part of the 0.2 m profile reported by the volumetric moisture reading to have been drying out between emergence and successive leaf appearance. This would lead to moisture stress of the young lucerne crop due to its shallow undeveloped root system. However, if this was the case a similar bilinear relationship would be expected in all sowing dates. Alternatively, the crop may have been using resources to develop a root system at the expense of leaf appearance as a result of undefined environmental signals prevailing at the time (Section 2.13). According to Fick *et al.* (1988), this lag phase is shortest under high temperatures that promote rapid leaf production and expansion. The shortest phyllochron ($P<0.001$) was observed in SD 2 (45 $^{o}$Cd) whilst the longest ($P<0.001$) was observed in SD 5 (65 $^{o}$Cd). This trend is consistent with Moot *et al.* (2001) who reported a systematically faster phyllochron in spring and summer (35 $^{o}$Cd per node) than in autumn (51 $^{o}$Cd per node) sown regrowth 'Grasslands Kaituna' crops. Brown *et al.* (2005) attributed the longer autumn phyllochron to a limited availability of assimilates to shoots due to a higher DM partitioning to crowns and roots as plants replenish reserves for overwintering and spring regrowth (Kim *et al.* 1991). The reverse is true for the faster phyllochron observed in SD 1. Belanger *et al.* (2003) purport that biomass partitioning to lucerne shoots is higher in spring than in summer.

Robertson *et al.* (2002) derived a mean phyllochron for seedling crops of 51 $^{o}$Cd ($T_b$=5 $^{o}$C) as a parameter for the APSIM-lucerne model. This was consistent with a mean of 54 $^{o}$Cd ($T_b$=5 $^{o}$C) reported in this study. Canopy expansion in SD 5 was slower than all other sowing dates due to ontogenetic factors triggered by changes in photoperiod (Section 2.13.3). Moot *et al.* (2003) observed a similar phyllochron of a single node appearing every 7-10 days on irrigated 'Grasslands Kaituna' in winter at mean temperatures of 4.5 $^{o}$C compared with every second day in summer at mean temperatures of 17.5 $^{o}$C. They attributed this to seasonal changes in the allocation of DM production between shoots and roots (Khaiti & Lemaire 1992). The differences in phyllochron observed between SD 4 and SD 5 (Table 4.2), despite similar mean temperatures (16.4 $^{o}$C) highlights seasonal changes in partitioning of assimilates from shoots to roots and suggests that factors other than temperature control leaf appearance (Teixeira *et al.* 2011) (Section 2.13). Brown *et al.* (2005) tested photoperiod as a predictor of phyllochron in regrowth lucerne, a mechanism thought to be triggered by the activity of genes below a critical photoperiod (Thies *et al.* 1992). Teixeira (2006) observed an increase in phyllochron

from 34 $^{o}$Cd in spring/summer to 65 $^{o}$Cd in autumn as the mean photoperiod decreased from 16 to 13.5 hours. Similarly, in this study the photoperiod declined from 15.2 to 14.2 from SD 3-5 whilst the respective seedling phyllochrons increased from 57-65 $^{o}$Cd. The slower leaf appearance rates in autumn sown lucerne have agronomic implications to crop establishment. In addition to reduced light interception, delayed canopy closure promotes weed growth. Winter annuals such as storksbill (*Erodium cicutarium*) or sub clover (*Trifolium subterraneum*) fill in the vacant gaps resulting from slow growth and reduced leaf appearance and eventually cause lucerne decline (Palmer 1982). Therefore additional weed control may be necessary for autumn sown crops.

## 5.6 Light interception

Regrowth crops had a 20% faster leaf area expansion rate (LAER) than seedling crops (0.006 ±0.0009) and thus attained $LAI_{crit}$ sooner (Figure 4.9). A steeper slope of the regression of LAER against Tt in regrowth crops compared with seedlings indicates that regrowth crops attained the highest $LAI_{crit}$ (4.0) earlier ($P<0.001$) and therefore had a shorter phyllochron. Seedling and regrowth crops showed a consistency of increasing fractional light interception with leaf area index despite moisture stress, although some variability was apparent. This suggests similarities in canopy architecture (Figure 4.9). Teixeira *et al.* (2011) previously reported a $LAI_{crit}$ of 3.6 in irrigated 'Grasslands Kaituna' lucerne, a figure consistent with findings from this study. Seedling and regrowth crops both calculated an extinction coefficient for incoming PAR (k) of 0.75 which demonstrates that mean canopy leaf angle and optical properties (canopy architecture) were unaffected by crop growth stage, seasons, defoliation frequency or the level of perennial reserves (Teixeira *et al.* 2008). This apparent lack of morphological change in canopy architecture despite an inconsistent moisture supply in this dryland study was also observed by Varella (2002) in irrigated lucerne subjected to full sun and severe shade. This can be explained by the planophile lower leaves and erectophile upper leaf arrangement across the lucerne canopy strata, a trait that enhances efficient tracking of solar radiation by the plant through its canopy architecture (Travis & Reed 1983). Thus, plasticity in leaf arrangement of lucerne would be unexpected because it is already an efficient canopy. This also suggests that lucerne has no drought avoidance strategies unlike sugarcane (*Saccharum officinarum* L.), for example which avoids water stress by reducing projected leaf area (Inman-Bamber & Smith 2005).

## 5.7 Time to reach bud initiation

According to Major *et al.* (1991) thermal time from defoliation to flowering decreases with increasing day length for lucerne. This was consistent with findings from SD 1 and 2 of this study which showed a requirement of 687 $^{o}$Cd at 13 hours and 651 $^{o}$Cd at 14 hour day lengths from emergence to flowering. SD 3 however did not conform to this trend and took 761 $^{o}$Cd at 15 hour day lengths. The delayed flowering in SD 3 would be expected under mild moisture stress conditions. Moot *et al.* (2001) reported an increase in thermal time requirement albeit at a much lower thermal time of 380-550 $^{o}$Cd as mean photoperiod decreased from 16 to 13.5 hours. The 550 $^{o}$Cd was consistent with Teixeira *et al.* (2011) for seedling and regrowth crops, thus delimiting a common basic vegetative period for flowering in a diverse group of lucerne cultivars (Section 2.13.4). SD 1 to 3 emerged in late spring/early summer, before the summer solstice, when photoperiod was still increasing. SD 4 and 5 in contrast emerged in late summer when photoperiod was beginning to decline. They consequently failed to flower despite adequate autumn soil moisture. This confirms that the thermal time requirement for flowering in lucerne is modified by photoperiod requirements (Moot *et al.* 2003). In response to declining photoperiods, SD 4 and 5 may have switched their preferential partitioning of DM from shoots to roots (Section 2.13.3) and in the process affected reproductive development. This therefore means that farmers need not use flowering as an indicator for grazing or defoliation if lucerne sowing is delayed till late summer. Seedling crops growing through autumn do not flower, thus upon appearance of the tenth leaf, grazing should commence. Due to the limited data points of sowing dates that flowered in this study, it is difficult to determine the existence of a juvenile phase independent of photoperiod through which a species must go through before floral initiation (Section 2.13.3).

## 5.8 Conversion of light into shoot biomass

The seedling growth of lucerne had an 18% lower pooled $RUE_{shoot}$ than the regrowth stage (Figure 4.10) despite having a similar crop yield of 450 g DM/m$^2$. This apparent reduction in conversion efficiency in seedling crops was consistent with Teixeira *et al.* (2011) who reported a 40% lower pooled $RUE_{shoot}$ in the seedling stage of 'Grassland Kaituna'. This has previously been attributed to a preferential allocation of assimilates to accumulate vegetative storage proteins and replenish carbon and nitrogen reserves in roots (Avice *et al.* 2003) (Section 2.13.1.1). Teixeira *et al.* (2011) also suggested a lower leaf photosynthetic capacity in seedlings as an alternative cause given the limited N supply from undeveloped roots still

forming nodules for $N_2$ fixation. This however may not apply to this study given the adequate mineral N levels reported at each sowing from soil test results (Table 3.1). Seedlings grow from seed with limited reserves sufficient only for germination and emergence. A lag period of leaf production before photosynthesis is therefore expected and can be longer depending on moisture availability. Avice *et al.* (2003) added that the absence of a root reserve pool limited N supply and subsequently $RUE_{shoot}$ in the seedling stage. Regrowth lucerne, depending on defoliation management, is able to remobilise and utilise perennial reserves to initiate growth faster than seedling crops (Brown *et al.* 2006). This initial advantage increases the $RUE_{shoot}$ of regrowth lucerne hence a higher annual production of 25 t DM/ha compared with 20 t DM/ha for lucerne seedlings (Brown *et al.* 2006).

# Chapter 6

# Genetic characterisation of bacteria

The ability to analyse the genetic constitution of rhizobial populations is of paramount importance in determining the relative nodulation capability of inoculants, indigenous rhizobia and the diversity of their populations (Gandee *et al.* 1999).

## 6.1 Non molecular techniques for microbial study

Before the advent of molecular analyses, traditional techniques for studying microbial populations were based on the cultivation of microbial populations, measurement of their metabolic activities and direct observation using microscopic techniques. Due to the variable nature of phenotypic characteristics, these techniques provided inadequate information on the genotypic characteristics of rhizobial strains and were strongly influenced by environmental factors (O'Callaghan & Gerard 2010).

Traditional techniques were also limited by; the need to culture rhizobia, the ability to follow only single or a few strains over time, labour intensity and the inability to characterise the nature of indigenous rhizobial populations (Thies *et al.* 2001). They focused on isolating bacteria from nodules or soil and testing them for their nitrogen-fixing ability on selected legume hosts. These isolates were initially ‘marked’ for distinction and ‘typed’ for further study by antibiotic resistance markers, multi-locus enzyme electrophoresis profiles, serological markers, biochemical markers or by protein profiles (Thies *et al.* 2001).

### 6.1.1 Antibiotic resistance markers

Antibiotic resistance markers, either intrinsic or induced, are the simplest method of identifying bacterial strains. They are economic (Thies *et al.* 2001), facilitate rapid data collection and are able to estimate the density of fast-growing rhizobia inoculated into non-sterile soil (Danso & Alexander 1973). Streptomycin, a commonly used antibiotic resistance marker, inhibits protein synthesis by mutating a ribosomal subunit and preventing it from

binding to an antibiotic. The altered molecules act as specific markers and distinguish strains through their ability to grow in the presence of an antibiotic (Wilson 1995). Bushby (1981) reported that antibiotic resistance markers naturally occur in rhizobial populations and thus may be limited in their discriminatory value. However, if induced they represent increased 'genetic load' which affects strain fitness in the field, thereby altering bacterial behaviour and observation. Wilson (1995) used the technique as an alternative to serology for identification and enumeration of the proportion of nodules formed by the inoculum strain under field conditions and noted labour intensity and the general lack of clarity in strain identification to be among its limitations (Wilson 1995).

### 6.1.2 Protein profiles

Protein profiles are an important technique through which rhizobial community diversity can be analysed (Moreira *et al.* 1993). Patterns derived from these analyses are however complex and challenging when used to distinguish closely related isolates. In addition, the technique is labour intensive in its execution and interpretation for use as a routine monitoring tool (Thies *et al.* 2001).

### 6.1.3 Multi-locus enzyme electrophoresis

Multi-locus enzyme electrophoresis or allozyme analysis is the standard technique for assessing rhizobial diversity, deriving estimates of strain relatedness and unravelling potential evolutionary pathways (Martínez-Romero & Caballero-Mellado 1996). According to Richardson *et al.* (1986) the technique does not require DNA extraction and is therefore simple in its execution. Information derived from it gains robustness with each allozyme analysed. It is however problematic when used as a routine tracking tool because small quantities of enzymes are inadequate for identification purposes and calculation of population diversity (Thies *et al.* 2001). According to Demezas *et al.* (1995) at least 12-20 enzyme loci may need to be examined for these analyses thereby making it a difficult technique for use on large-scale field studies.

### 6.1.4 Polyclonal antibodies

Polyclonal antibodies are often prepared specifically for strains of interest in inoculation programs (Thies *et al.* 2001). While many such antisera may cross-react with closely related strains, techniques such as cross absorption have been used to increase their specificity (Hoben *et al.* 1994). Regardless, antisera are limited to identification of selected strains and tell investigators relatively little about the rhizobial community into which introduced strains are placed. The possibility also exists for bacterial strains to alter cell surface markers over time, particularly under high selection pressure (Johnsson *et al.* 1998), which may render specific antisera of limited value. For some species, such as *E. meliloti*, cross reaction of antisera is so common that only monoclonal sera have proved useful and even these have been of limited value (Thies *et al.* 2001).

## 6.2 Molecular techniques for microbial study

Molecular methods were developed to address the shortcomings of traditional techniques. They are relatively easy to use, they lack the need to culture bacteria before analysis and they provide information and distinguish between all members of a given community. In addition, they enable tracking of chromosome and plasmid located genes, which should lead to new insights into the mechanisms by which strain diversity develops in soils over time. Molecular methods, particularly polymerase chain reaction (PCR), have revolutionised the study of microorganisms *in situ* (Mullis *et al.* 1987).

### 6.2.1 Polymerase chain reaction

The polymerase chain reaction (PCR) can be used for both identification and classification of individual rhizobia isolated from field-grown, inoculated plants (Hebb *et al.* 1998). It is a useful tool for examining practical establishment and persistence problems of inoculant rhizobia introduced at sowing. Rhizobial identification by PCR gives an image of a soil with regard to its diverse population pool of naturalised rhizobia and provides information regarding the relatedness between inoculant strains and field isolates. Using PCR amplification profiles for strain identification is a powerful complement to other means of assessing the effectiveness of rhizobia to host legumes, as in the enumeration of rhizobial populations and physical measurements of indicators of $N_2$ fixation. High levels of discrimination in PCR-fingerprinting make it possible to identify or ‘benchmark’ inoculant

strains and thereby confirm unknown rhizobial isolates as inoculant strains (Hebb *et al.* 1998). It also tracks rhizobial strain distribution and spread, characterises site populations, monitors genetically modified rhizobia in field soils (Cullen *et al.* 1998) and assess the outcomes of competition between strains (Niemann *et al.* 1997).

PCR involves separation of a double-stranded DNA template into two strands, the hybridisation of primers (oligonucleotides) to the template and then the elongation of the primer-template hybrid by a polymerase enzyme (Mullis *et al.* 1987). It makes infinite numbers of copies of any piece of DNA. The target DNA segment can be amplified by using a combination of short pieces of DNA which border the segment of interest and bind to their complementary strand in the target DNA (primers). The PCR mix is composed of a heat stable polymerase enzyme such as *Taq* polymerase, free deoxynucleotides (A, T, G and C; the building blocks of the new DNA strands), a buffer which provides the optimum pH and salts for enzyme action, the two PCR primers and a little amount of the DNA to be amplified. The DNA is multiplied through a three stage temperature dependent process. Firstly the DNA is denatured into single strands at 94 $^{o}$C. Secondly, the reaction is then cooled down to ~45-60 $^{o}$C allowing the primers to anneal to perfectly complementary target sites on each of the two single DNA strands. Extension of strands begins and progresses into the last phase where the temperature is raised to 72 $^{o}$C which is optimal for the Taq polymerase and extension allowing DNA polymerases to build a new strand from the bound primer. This process builds new copies of the specific piece of DNA between the primers such that by the 35$^{th}$ cycle of the three stages, 68 billion copies of the target sequence are achieved. This enables visualisation of the target piece of DNA from the background genome that was not amplified (Glare & Ridgway 2010).

According to Jensen *et al.* (1993) primers were generally developed to facilitate genus and species level identification of a broad range of bacteria without the requirement of presumptive identification. 16S ribosomal RNA (rRNA) genes and 16S-23S rRNA intergenic spacer regions are most commonly targeted primers in most *Rhizobium* ecological studies (Gürtler & Stanisich 1996). 16S genes are designed to target specific DNA fragments or genes for nitrogen fixation and nodulation (Thies *et al.* 2001). Alternatively they target repetitive sequences such as the interspersed repetitive DNA (BOX) sequences known for their potential to form stable stem-loop structures located in the immediate vicinity of genes (Martin *et al.* 1992). Repetitive extragenic palindromic (REP) sequence and enterobacterial repetitive intergenic consensus sequences are also targeted, mainly because they produce clearly

resolvable bands by agarose gel electrophoresis thus enabling rapid identification of bacterial species and strains (Thies *et al.* 2001).

ERIC primers have been used by *Rhizobium* researchers to obtain 'PCR-fingerprints' which are used to characterise rhizobial isolates at the strain level (Niemann *et al.* 1999). Arbitrary primers generate randomly-amplified polymorphic DNA (RAPD) fragments from a wide variety of species which are analysed by gel electrophoresis to provide a "fingerprint" profile for any particular target genome (Williams *et al.* 1990). They are well suited for genetic mapping in plant and animal breeding applications and DNA fingerprinting. RAPD markers also provide an efficient assay for polymorphisms enabling rapid identification and isolation of chromosome-specific DNA fragments (Williams *et al.* 1990). They are however limited in that the fingerprint patterns obtained may vary due to subtle variations in PCR conditions (Gillings & Holley 1997).

Niemann *et al.* (1999), in a study of the genetic basis of ERIC-PCR in *E. meliloti,* established that repetitive ERIC or ERIC-like sequences are not an integral part of the *E. meliloti* genome. Instead the ERIC2 primer binding sites in the *E. meliloti* genome are predominantly located in protein coding regions unlike in *Escherichia coli,* where they are located in intergenic, non-coding regions. ERIC-PCR fingerprinting in *E. meliloti* and other bacterial species is therefore similar to the RAPD method which employs short primers of a random sequence in PCR reactions to generate strain specific fingerprints (Gillings & Holley 1997).

In a study to determine the existence of REP and ERIC-like sequences in the genomes of *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Agrobacterium* and *Pseudomonas*, de Bruijn (1992) reported these sequences to be highly conserved in *Rhizobia*, *Agrobacteria*, and *Pseudomonas*. As a result, he recommended the use of REP and ERIC PCR to distinguish and classify closely related Rhizobium strains of widely divergent geographic origins. Hebb *et al.* (1998) monitored field establishment and persistence of inoculants of *R. Leguminosarum* bv. *trifolii* and *E. meliloti* in 10 clover (*Trifolium* spp.) and 8 annual *Medicago* spp. by use of PCR amplification profiles. In the second season, nodule isolates characterised from each species yielded a mean recovery of 53% for *Medicago* spp and 45% for clovers across 8-10 hosts respectively. This validated PCR utility in enabling rapid identification of inoculant strain types in a range of host plants (Allen & Allen 1958).

#### *6.2.1.1 Electrophoresis of nucleic acids*

Amplified PCR products are often visualised by running through an electrophoretic gel which is then stained with ethidium bromide (Thies *et al.* 2001). Agarose, despite poor resolution, is the most common medium for electrophoretic separation of medium and large-sized nucleic acids due to its large working range. The electric current in standard electrophoresis can separate nucleic acids between 0.1 and 70 kb in size depending on the agarose concentration used (Thies *et al.* 2001). It forces the molecules through pores in the gel, which results in separation depending on size, shape and electrical charge of the DNA and RNA fragments (Glare & Ridgway 2010). Analysis of the amplification products is based on the presence and pattern of DNA bands in the gel matrix. From these bands it is possible to estimate the size of DNA fragments in gels by running fragments of known size on the same gel (Glare & Ridgway 2010).

## 6.3 Genotypic characterisation of nodules collected from the field trial

### 6.3.1 Collection and sterilisation of nodules

From the field site, plants were selected from each of the four treatments of sowing date 2 between the 7$^{th}$ and 14$^{th}$ of January 2011 at which stage the plants were 10 weeks old. A total of 20 plants were randomly selected per treatment, across random plots and uplifted. The plant roots were washed thoroughly in tap water to remove excess soil. From the crown of the main tap root of each plant, 5–7 pink coloured nodules (~60 nodules per treatment) were removed for genotyping. Each nodule was recovered by cutting the root at a distance of approximately 5 mm on either side of the nodule using a flame sterilised scalpel and tweezers. The nodule and remaining root fragment was then surface sterilised by immersion in 95% ethanol for 5–10 sec, 20% bleach for 2 min and then rinsed four times in sterile water for 20 sec each time.

### 6.3.2 Recovery of bacteria from the sterile nodules

In the laminar flow cabinet the surface sterilised nodules were then detached from the root fragments with a sterile scalpel, crushed with the flattened end of a flamed glass rod in a sterile Petri dish, and a loopful of the bacterial contents plated with a loop onto yeast mannitol

agar (YMA; 0.1% (w/v) yeast extract, 1% (w/v) mannitol, 0.0005 mM dipotassium phosphate, 0.0002 mM magnesium sulphate, 0.0001 mM sodium chloride, 0.0005 mM calcium carbonate, 1.5% (w/v) agar, autoclaved 15 min, 121°C, 15 Psi ). This process was repeated for all nodules from each treatment. The YMA plates were incubated at 20 ºC for 48 h in the dark. Cultures were sub-cultured at least twice to ensure purity. Once pure cultures (assessed by visual examination) were obtained from the selected sub cultures, based on the morphological characteristics of the rhizobia, they were stored on the YMA plates at 4 °C prior to DNA extraction.

### 6.3.3 Recovery of bacteria from commercial inoculants

Ten 'Stamina 5' lucerne plants were also grown in an Adaptis (Conviron) incubator using the sterile seedling method described by Weir (2006) to grow and extract bacteria from the commercial inoculants (ALOSCA®, peat and coated seed). The sterile seedling method involved surface sterilization of the seeds in 5% bleach for 5 min and then rinsing in sterile water 2-3 times. One seed was placed in the centre of a water agar plate and left to germinate in the dark (to encourage root growth) for up to 3 days. One seed was used per treatment including the negative control. Approximately 40 ml of sterile nutrient solution was added to sterile dry vermiculite (70–100 mm depth) contained in sterile plastic bottles in the laminar flow. A single seedling was transplanted into each of the 10 bottles (3 per treatment and 1 control). (20% v/v) sterile water was added to keep the vermiculite moist without over or under watering. A nutrient solution was used (Appendix 2) (Andrews 2011) to provide essential micro nutrients for the healthy establishment of the seedlings.

Inoculation of seedlings occurred at transplanting (Section 3.4). A 10% slurry was made for ALOSCA® and peat by dissolving 20 g of inoculant in sterile distilled water (SDW). The slurry was mixed on a shaker for 30 min and 5 ml was applied to the base of each plant. For coated seed, 55 g of seed were shaken in 100 ml of SDW for 30 min. The lime coat solution was then diluted 10 times before 5 ml were applied to the base of the plant. In the control 5 ml of sterile water were applied instead of inoculum. Plants were irrigated weekly and left to grow for 2-4 weeks in the Adaptis (Conviron) incubator at 22 °C, for 12/12 h light/dark per day. Developed nodules were then harvested and the bacteria isolated and genotyped (Section 6.2). Banding patterns from the commercial inoculants were compared with those from the nodule isolates to determine if the inoculants were responsible for nodulation in the field.

### 6.3.4 DNA isolation from rhizobia

A single loopful of each bacterial colony was removed from the stored YMA plate and used to inoculate 1 mL of yeast mannitol broth (YMB; 0.1% (w/v) yeast extract, 1% (w/v) mannitol, 0.0005 mM dipotassium phosphate, 0.0002 mM magnesium sulphate, 0.0001 mM sodium chloride, autoclaved in a sterile 2 mL tube. This liquid suspension was incubated in a shaking incubator at 28 ºC for 24-48 h at 220 rpm (LABNET 211 DS, Labnet International, USA). Control tubes which contained YMB but were not inoculated were included in each batch to check for the presence of contamination. DNA was extracted from the rhizobial cultures using the PUREGENE™ (Gentra Systems, USA) DNA extraction kit according to the manufacturer's instructions as follows. The 1.2 mL broth culture was centrifuged at 16,000 ×g for 2 min to pellet the bacterial cells and the supernatant discarded. To the cell pellet 300 µL of cell lysis solution was added, the pellet fully re-suspended by pipetting and then the solution incubated at 70 °C for 5 min. One µL of RNase A Solution (Invitrogen) was added to cell lysate and mixed by inverting the tube 25 times before incubating at 37 $^{o}$C for 15 min.

The sample was cooled to room temperature and 100 µL of protein precipitation solution was added to cell lysate and uniformly mixed for 15 s by vortexing. The solution was then centrifuged at 16,000 ×g for 3 min to form a tight pellet of precipitated proteins and cell debris at the base of the tube. The supernatant containing the DNA was transferred into a clean 1.5 mL tube and precipitated with 300 µL of ice cold 100% isopropanol (2-propanol) and mixed by inverting gently 50 times. The DNA was pelleted by centrifugation at 16,000 ×g for 1 min. The supernatant was discarded and the DNA pellet was washed by adding 500 µL of 70% ethanol and inverting the tube several times before centrifuging again at 16,000 ×g for 1 min and carefully pouring out the ethanol. The tube was then drained on clean absorbent paper and allowed to air dry for 10-15 min.

#### *6.3.4.1 DNA Hydration and Spectrophotometry*

The DNA pellet was rehydrated by adding 30 µL of sterile water and leaving samples overnight at room temperature prior to short term storage at 4$^{o}$C (resulting in a DNA concentration of between 100–300 ng/µL). 1 µL of DNA was quantified and qualified using a Nano Drop at wavelengths of 260 and 280 nm.

### 6.3.5 PCR amplification of rhizobial DNA using ERIC primers

DNA fingerprinting of the recovered bacterial colonies was done by polymerase chain reaction (PCR) with primers ERIC 1R (5'-ATGTAAGCTCCTGGGGATTCAC-3') and ERIC 2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') (Versalovic *et al.* 1991). Each 25 µL PCR reaction contained 2.5 µL of 10X buffer (FastStart, Roche, USA), 200 µM of each dNTP, 50 pmole of each primer, 1.25 U *Taq* DNA polymerase (Roche FastStart) and 1 µL of extracted DNA (50 ng/µL). The DNA was amplified in a PCR thermal cycler with the following thermal cycle; 94$^{o}$C for 3 min to allow denaturation, then 35 cycles of: 94$^{o}$C for 30 s (denaturation), 52$^{o}$C for 90 s (annealing) and 72$^{o}$C for 8 s (extension), with a final elongation cycle of 72$^{o}$C for 7 min.

### 6.3.6 Electrophoresis

The resultant PCR products were separated and visualised by electrophoresis on a 1% agarose gel at 10 V/cm for 50 min in 1× TAE buffer (18 mL distilled $H_2O$, 0.484% (w/v) tris base, 1.142 mL glacial acetic acid, 0.0372 % (w/v) EDTA). In this process 5-8 µL of the PCR product was mixed with 2 µL of loading dye (0.025% bromophenol blue, 0.025% xylene cyanol, 40% w/v sucrose), and pipetted into agarose wells. In the first lane of each gel 8 µL of 1kb Plus DNA ladder (Invitrogen) was loaded. The gels were stained by immersion for 15 min in ethidium bromide (0.5 µg/L) and then destained for 5 min in sterile water. The stained gel was visualised under UV light using the Versadoc 3000™ (BioRad) and images captured using Quantity 1™ (BioRad) software.

### 6.3.7 Band scoring/genotyping

Gel images from each of the 200 treatment isolates were visually assessed and scored to identify each of the distinct bacterial genotypes present. Samples showing identical banding patterns, regardless of treatment, were assumed alike and allocated the same genotype code. The seven most common genotypes across all treatments were then noted and individual treatment genotypes recorded.

### 6.3.8 Amplification of 16S ribosomal DNA for isolate identification

The 16S rDNA gene of the seven most common genotypes (A, M, B, J, I, C and L) was amplified for DNA sequencing. Each 25 µL PCR reaction contained 2.5 µL of 10 × buffer (FastStart, Roche, USA), 200 µM of each of the dNTPs, 0.25 U *Taq* DNA polymerase (FastStart, Roche, USA) and 10 pmole of each of the primers F27 (5'AGAGTTTGATC (A/C)TGGCTCAG-3') and R 1494 (5'CTACGG(T/C)TACCTTGTTACGAC-3') (Gomes *et al.* 2001; Weisburg *et al.* 1991) (Invitrogen). 1 µL of the DNA template diluted down to 20-25 ng/µL was added to each tube. A negative control containing all reagents but the DNA template was included. Microtubes containing the reaction mix were amplified in a thermal cycler (Veriti™, Applied Biosystems, California, USA) under the following protocol: 94 °C for 3 min (denaturation), then 35 cycles of: 94 °C for 30 s (denaturation), 55 °C for 30 s (annealing) and 72 °C for 1 s (extension), and a final cycle of 72 °C for 7 min. The resulting PCR products were then loaded onto 1% agarose gels and the DNA separated by agarose gel electrophoresis as described in Section 6.2.1.

### 6.3.9 DNA sequencing

The amplified isolates (Section 6.2.1) were then sequenced to obtain genus or species level identification. Sequencing proceeded in one direction with primer R1494 (Invitrogen), at the Lincoln University Sequencing Facility using an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems, California, USA). Chromas Lite 2.1 (Technelysium Pty Ltd, Australia) was used to view Sequence electropherograms which were then manually trimmed using DNAMAN 4.0 (Lynnon Biosoft, Canada). The sequences obtained were compared with those of known origin using the nucleotide database and the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.* 1990).

## 6.4 Genotypic characterization of rhizobia from lucerne plants

Genotypic characterization was performed on 50 bacterial isolates from 50 'Stamina 5' lucerne nodules extracted from 20 randomly selected plant treatments at flowering (Section 6.3.1).

### 6.4.1 Bare seed (Control)

Based on the unique ERIC-PCR banding patterns observed, 11 different genotypes were identified (Plate 6.1) for the un-inoculated bare seed control.

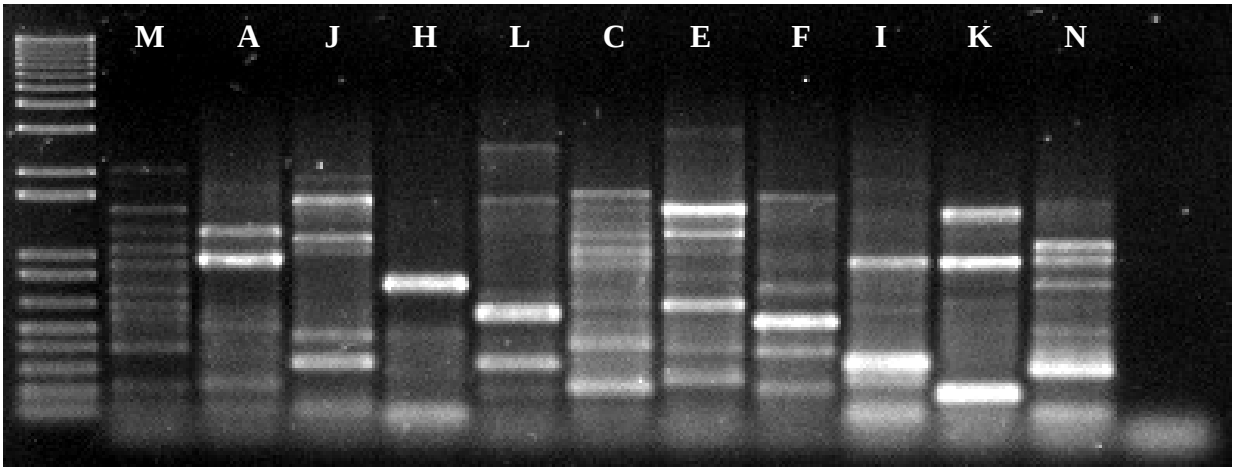


**Plate 6.1 Agarose gel illustrating the 11 distinct ERIC-PCR banding patterns obtained from DNA isolates recovered from un-inoculated bare seed 'Stamina 5' lucerne plants that were harvested for nodule collection in January 2011 at Lincoln University, Canterbury. Lane 1 contains the 1Kb Plus DNA Ladder™ (Invitrogen) and Lane 13 is the non template control.**

Some bacterial genotypes occurred more frequently than others in the bare seed treatment. Genotype M was the most common and was present in 28% (14/50) of the isolates (Figure 6.1). Genotypes A, J, H and L also appeared quite frequently at 16%, 12%, 10% and 8%, respectively. Genotypes C and E-N were less common at 6% and 4% respectively with just three and two genotypes represented in the 50 isolates sampled for bare seed.

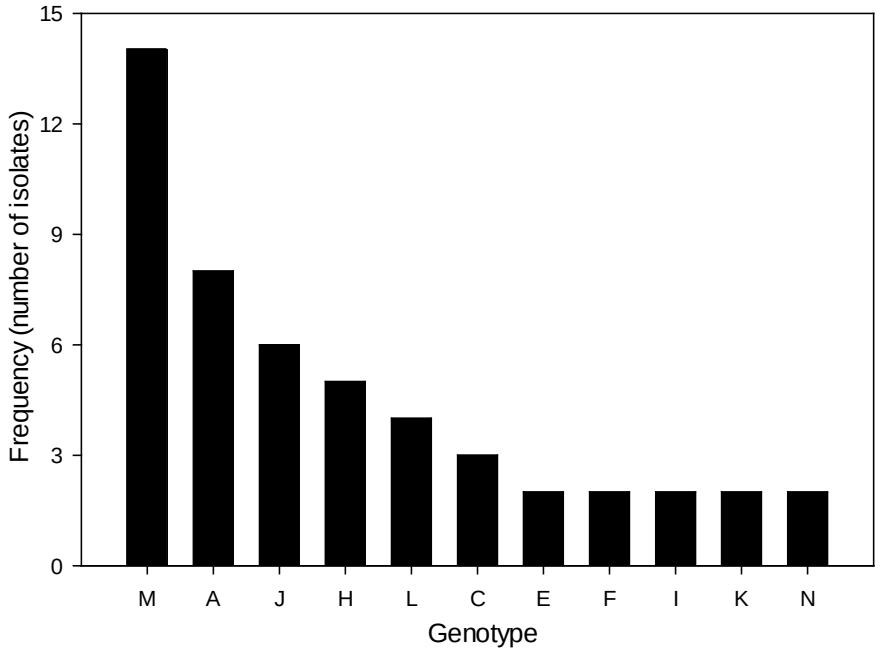


**Figure 6.1 Frequency of the 11 bacterial genotypes (Plate 6.1) found in the nodules of un-inoculated bare seed 'Stamina 5' lucerne plants sown on the 5th of November 2010 and harvested for nodule collection in January 2011 at Lincoln University Canterbury.**

### 6.4.2 ALOSCA®

Based on the unique ERIC-PCR banding patterns observed, 12 different genotypes were identified from ALOSCA® treated plants (Plate 6.2).

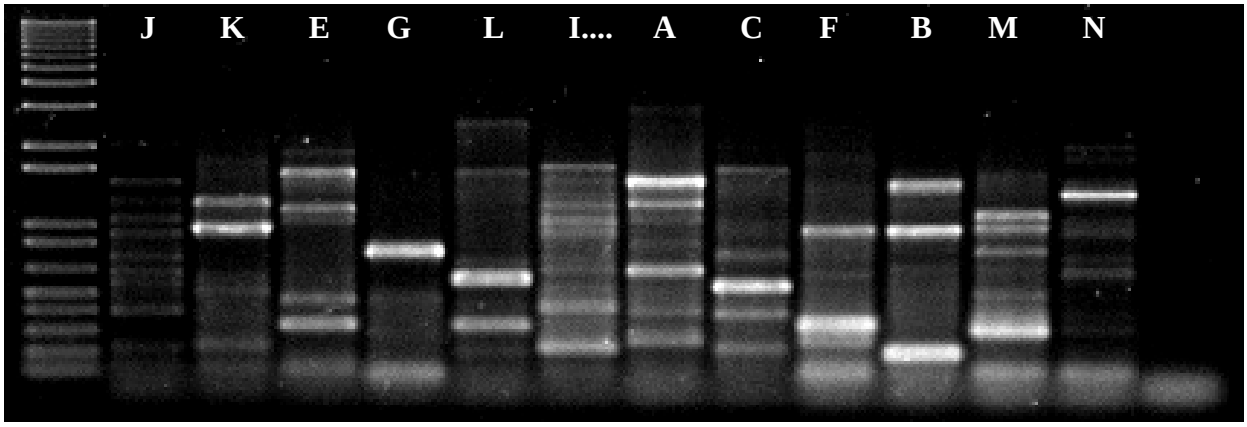


**Plate 6.2 Agarose gel illustrating the 12 distinct ERIC-PCR banding patterns obtained from DNA isolates recovered from ALOSCA® treated 'Stamina 5' lucerne plants that were harvested for nodule collection in January 2011 at Lincoln University, Canterbury. Lane 1 contains the 1Kb Plus DNA Ladder™ (Invitrogen) and Lane 14 is the non template control**

Figure 6.2 shows the most common genotypes J and K at a frequency of 16% (8/50) out of the 50 isolates from ALOSCA® treatments. Genotypes E, G and L were present in seven (14%) of the isolates and genotype I, A and C were present in four, three and two respectively (8%, 6% and 4%). Genotypes M, N and B were the least common in the 50 ALOSCA® isolates sampled with just one of each genotype present in the isolates recovered.

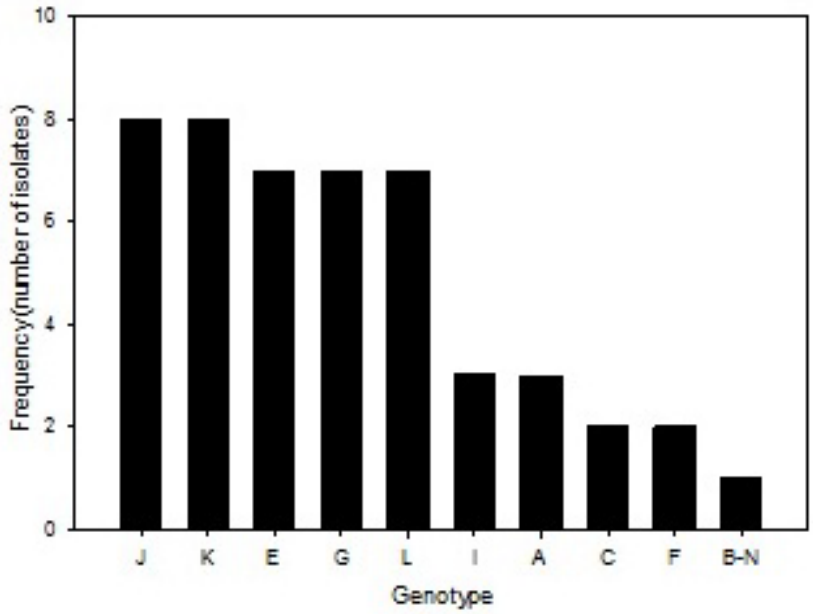


**Figure 6.2 Frequency of the 12 bacterial genotypes (Plate 6.2) found in the nodules of ALOSCA® treated 'Stamina 5' lucerne plants sown on the 5th of November 2010 and harvested for nodule collection in January 2011 at Lincoln University Canterbury.**

### 6.4.3 Coated seed

Nine different genotypes were identified from lime coated treatments based on the unique ERIC-PCR banding patterns observed (Plate 6.3).

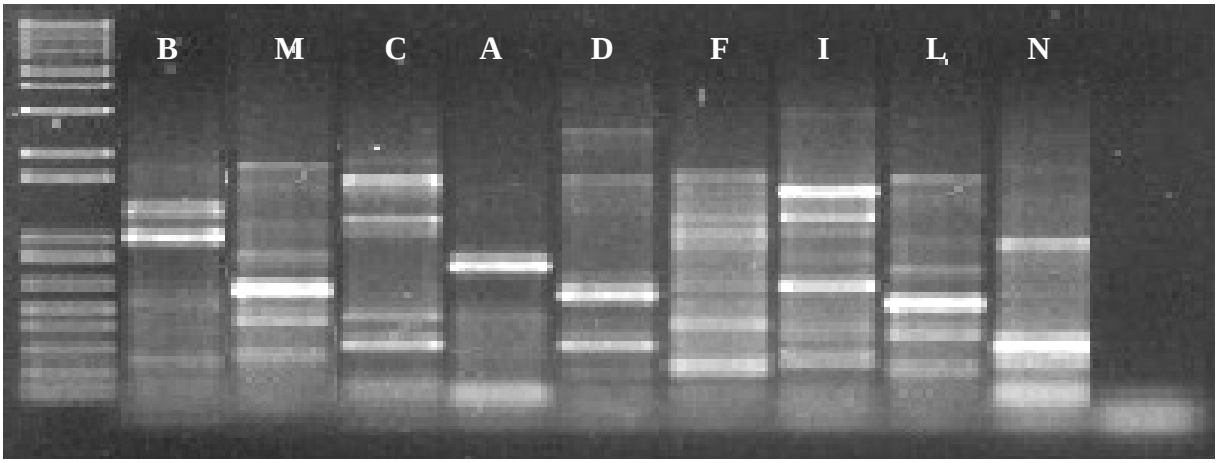


**Plate 6.3 Agarose gel illustrating the 9 distinct ERIC-PCR banding patterns obtained from DNA isolates recovered from lime coat treated 'Stamina 5' lucerne plants that were harvested for nodule collection in January 2011 at Lincoln University, Canterbury. Lane 1 contains the 1Kb Plus DNA Ladder™ (Invitrogen) and Lane 11 is the non template control**

Some bacterial genotypes occurred more frequently than others in the coated seed treatment. Genotype B was the most common and was present in 40% (20/50) of the isolates (Figure 6.3). Genotype M was the second most common at a frequency of 26% (13/50) of the isolates. Genotype C was present in 7 (14%) of the isolates whilst genotypes A and D were present in 3 (6%) of the isolates. Genotypes F, I, L and N each occurred just once and were the least common genotypes from the 50 isolates sampled for coated seed.

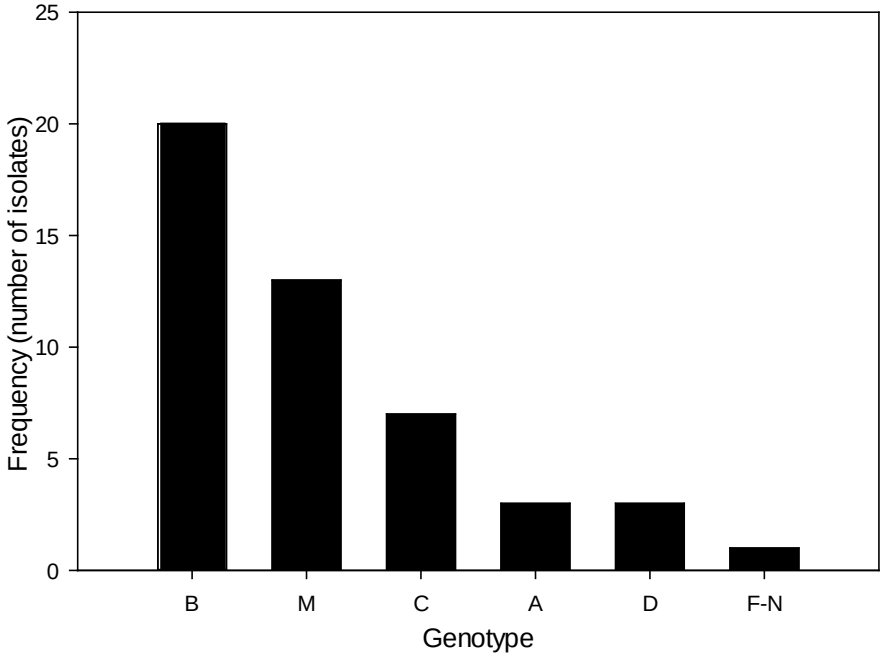


**Figure 6.3 Frequency of the nine bacterial genotypes (Plate 6.3) found in the nodules of the lime coated 'Stamina 5' lucerne plants sown on the 5th of November 2010 and harvested for nodule collection in January 2011 at Lincoln University Canterbury.**

### 6.4.4 Peat seed

There were 11 distinct genotypes identified in Plate 6.4 based on the unique ERIC-PCR banding patterns observed.

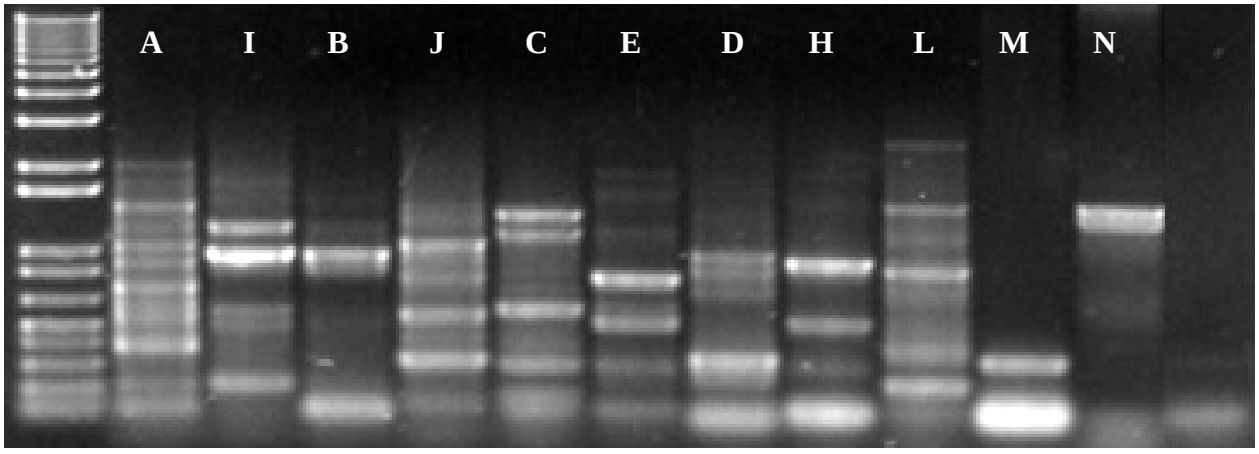


**Plate 6.4 Agarose gel illustrating the 11 distinct ERIC-PCR banding patterns obtained from DNA isolates recovered from peat treated 'Stamina 5' lucerne plants that were harvested for nodule collection in January 2011 at Lincoln University, Canterbury. Lane 1 contains the 1Kb Plus DNA Ladder™ (Invitrogen) and Lane 13 is the non template control.**

Some bacterial genotypes occurred more frequently than others in the peat seed treatment. Genotype A was the most common and was present in 30% (15/50) of the isolates (Figure Figure 6.4). Genotype I was the second most common at a frequency of 22% (11/50) of the isolates. Genotypes B, J, C and E were present in 8 (16%), 5 (10%), 4 (8%), and 2 (4%) respectively. Out of the 50 isolates sampled, genotypes D, H, L, M and N were the least common with just one of each genotype present in the isolates sampled from the peat seed treatment.

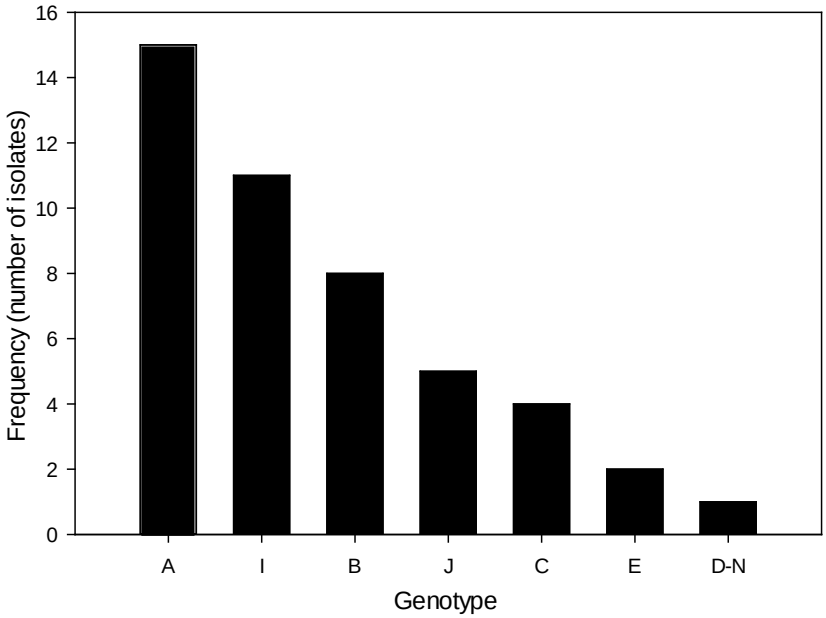


**Figure 6.4 Frequency of the 11 bacterial genotypes (Plate 6.4) found in the nodules of peat treated 'Stamina 5' lucerne plants sown on the 5th of November 2010 and harvested for nodule collection in January 2011 at Lincoln University, Canterbury.**

## 6.5 Overview

Genotype A (*Rhizobium tibeticum*), was the most commonly occurring genotype across all treatments including the bare seed control (Table 6.1). The second most common was Genotype B (*Ensifer meliloti*) found mostly in the lime coated (40%) and peat treatments (14%). It appeared less frequently in the ALOSCA® treatment (2%) and was absent altogether in the bare seed control. Genotype M was identified as *Pseudomonas* spp and was more common in the bare seed control (26%) than all other treatments. The remaining four genotypes were less frequent in occurrence and are listed in Table 6.1.

**Table 6.1 Occurance of the seven most common genotypes observed from isolates of nodules extracted from 'Stamina 5' lucerne plants treated with a bare seed control (BS), ALOSCA® (AS), lime coating (CS) or peat inoculant (PS) and sown on the 5th of November 2010 and harvested for nodule collection in January 2011 at Lincoln University, Canterbury.**

| | | Treatment | | | | |
|---|---|---|---|---|---|---|
| **Genotype** | **Name/genus/species** | **BS** | **AS** | **CS** | **PS** | **Total** |
| **A** | *Rhizobium tibeticum* | 8 | 3 | 3 | 15 | 29 |
| **B** | *Ensifer meliloti* | 0 | 1 | 20 | 7 | 28 |
| **M** | *Pseudomonas* sp. | 13 | 1 | 11 | 1 | 26 |
| **J** | *Rhizobium tibeticum* | 6 | 8 | 0 | 5 | 19 |
| **I** | *Rhizobium* sp. | 2 | 3 | 1 | 11 | 17 |
| **C** | *Rhizobium* sp. | 3 | 2 | 7 | 4 | 16 |
| **L** | *Pseudomonas corugata* | 4 | 7 | 1 | 1 | 13 |

## 6.6 DNA sequencing of the seven most common genotypes

The seven most common genotypes isolated were successfully identified to genus level using BLAST. All 16S sequences generated had at least 99% similarity when compared with genera in the GenBank database illustrated in Table 6.2. DNA sequences used for similarity matching ranged in size from 575-787 base pairs (bp). Genotype B isolates had 100% identity to *Ensifer meliloti* whilst genotypes A and H isolates were identified as *Rhizobium tibeticum.* Genotypes M and L matched with two different *Pseudomanas* sp. whilst genotypes I and C were both identified as plant *Rhizobium* sp.

**Table 6.2 (16S) sequences from representatives of the seven most common genotypes compared with known genera using BLAST. DNA was characterized from isolates recovered from the nodules of 'Stamina 5' lucerne plants treated with a bare seed control ALOSCA®, lime coating or peat inoculant at Lincoln University, Canterbury 2011. Only the highest matches from GenBank are illustrated.**

| Genotype | Name/genus/species | Source | Accession No. | % Coverage | % Identity | Product size (bp) |
|---|---|---|---|---|---|---|
| **A** | *Rhizobium tibeticum* | Soil | FR714442.1 | 100 | 100 | 620 |
| **B** | *Ensifer meliloti* | Plant | JN685309.1 | 100 | 100 | 575 |
| **M** | *Pseudomonas sp.* | Legumes | JF834141.1 | 100 | 99 | 675 |
| **J** | *Rhizobium tibeticum* | Soil | FR714442.1 | 99 | 100 | 775 |
| **I** | *Rhizobium sp.* | Plant | FR714442.1 | 100 | 100 | 582 |
| **C** | *Rhizobium sp.* | Plant | FR714442.1 | 100 | 99 | 620 |
| **L** | *Pseudomonas corugata* | Soil | JN638054.1 | 99 | 99 | 787 |

## 6.7 Genotypic characterisation and DNA sequencing of rhizobia extracted from commercial inoculants.

Bacterial isolates with a white, glossy or creamy viscous morphology resembling that of rhizobia were genotypically characterized. Four nodules per treatment from separate incubated plants grown in an incubator (Section 6.3.3) were harvested and inoculated with each of the treatments; ALOSCA®, lime coating, peat and a bare seed control. Based on the unique ERIC-PCR banding patterns observed, all three seed treatments contained the same genotype identified as B (Plate 6.5).

Bacterial isolates, recovered from the nodules of plants inoculated *in vitro* with three different treatments, were successfully identified using BLAST. A comparison of the 16S generated sequences with those stored in the GenBank gave a 99% similarity (as indicated by the maximum identity value) and 100% sequence coverage. DNA sequences used for similarity matching were 830 bp in length. All three isolates identified as genotype B from the different treatments were confirmed to be *Ensifer meliloti.*

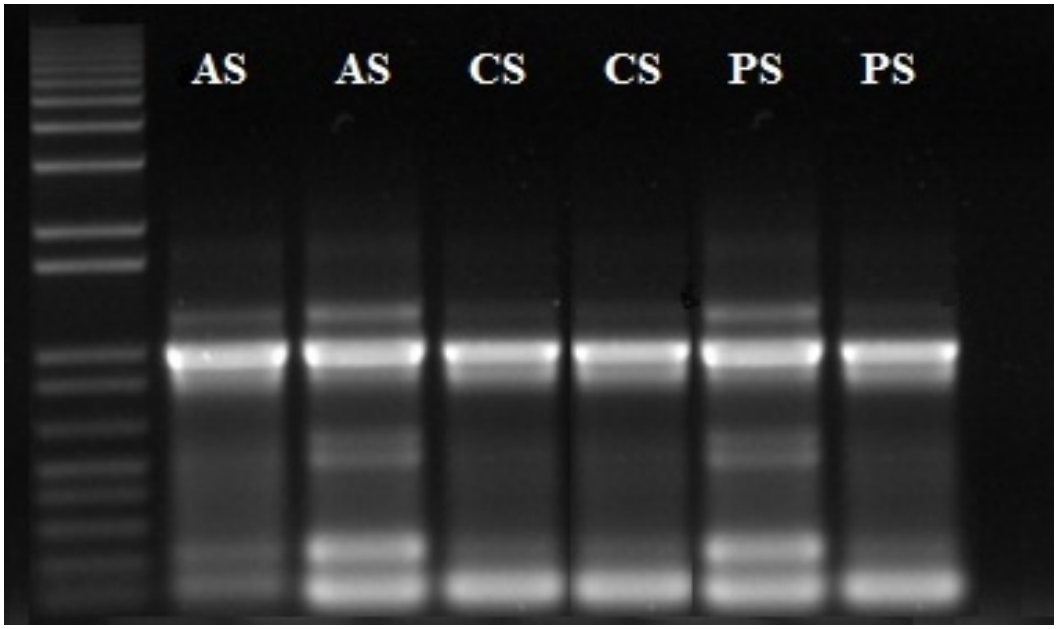


**Plate 6.5 Agarose gel illustrating ERIC-PCR banding patterns obtained from DNA isolates recovered from ALOSCA®, lime coating and peat peat treated 'Stamina 5' lucerne plants grown in sterile vermiculite at Lincoln University, Canterbury. Lane 1 contains the 1Kb Plus DNA Ladder™ (Invitrogen) and Lane 8 is the non template control.**

## 6.8 Discussion

A variety of genotypes were found in the bacterial isolates collected from the bare seed control, ALOSCA ® granules, lime coating and peat inoculant treatments. Between 9 and 11 genotypes were found per treatment (Plates 6.1-6.4), seven of which occurred more frequently than the rest (Table 6.1). DNA sequencing of these identified three different *Rhizobium* strains and two *Pseudomonas* species (Table 6.2). Genotype B was identified as *Ensifer meliloti* and occurred in 40% of coated seed isolates, 14% of peat seed isolates and 2% of ALOSCA® isolates. It was, however, not found in the bare seed control isolates suggesting that it was not an indigenous strain resident in the soil. ERIC-PCR fingerprinting and sequencing of the 16S rDNA region of the ALOSCA ® granules, lime coating and peat inoculant isolates also identified the seed treatments as *Ensifer meliloti* and therefore, the most obvious origin of genotype B found in the nodule isolates.

Plant associated *Pseudomonas* species are rhizospheric bacteria with the ability to colonize root surfaces and stimulate plant growth (Rodríguez-Navarro *et al.* 2007). They show plant growth-promoting activity through bio-control and may live as pathogenic parasites on plant

surfaces and inside plant tissues (Somers *et al.* 2004). The occurrence of *Pseudomonas* spp. as the dominant (14.5%) genotype amongst treatment isolates in this study could therefore be a result of its incidence in the soil rhizosphere. Alternatively, it may also have been a consequence of contamination from soil particles attached to root nodules during bacterial isolation. This is however very unlikely given the surface sterilisation of nodules done with 5% commercial bleach (Section 6.3.3) in the laminar flow to eliminate any contaminants from the surface of the nodules. A review by Hallmann *et al.* (1997) identified *Pseudomonas* as one of the most commonly isolated bacterial genera in leguminous plants. This suggests that rather than through contamination, the *Pseudomonas* found dominant in this study may have been endophytic within the nodules.

In addition to the *Ensifer meliloti* found in genotype B (14%), Genotypes A (8%) and H (6.5%) were identified as *Rhizobium tibeticum*. According to Carter *et al.* (1995) indigenous rhizobia vigorously compete with introduced strains and are often more successful at nodulation. Thus the occurrence of *Rhizobium tibeticum* in the root nodules may indicate that it is the indigenous rhizobia resident in the soil at the experimental site. Genotype B (*E. meliloti*) was present in 40% of the coated seed isolates and was dominant over genotypes M (22%), C (8%) and A (6%) in the same treatment. It also appears to have been more dominant in coated seed than in peat (14%) and ALOSCA ® (2%), a phenomena that can be ascribed to the properties of the lime coating (Section 2.6.2). Caetano-Anollés *et al.* (1989) demonstrated that adhesion of *E. meliloti* to lucerne roots required a neutral pH. This may explain the advantage coated seed had over the other treatments depending on the specific pH of the rhizosphere at the time of sowing. The lime contained in the coating neutralizes pH anomalies and creates a favourable environment for rhizobial nodulation. *E. meliloti* was less dominant (14%) over *Rhizobium tibeticum* (30%) and *Pseudomonas corugata* (22%) in the peat isolates. This is probably because peat inoculum is easily contaminated and generally endures consistent unsatisfactory nodulation of new stands particularly when dry weather prevails (Wynn-Williams 1976) (Section 2.6).

During preparation of the peat slurry, despite the strict asceptic protocols observed, contamination from free floating bacteria is still highly likely and so is the possibility of peat inoculum drying out and shaking off the bare seed and severely reducing the total viable rhizobial count per seed. According to Wynn-Williams (1976) the acidic nature of most peat deposits reduces the rhizospheric pH (Section 2.6.1) making it unfavourable for rhizobial survival and subsequent nodulation.

A diverse range of genotypes were recovered from the ALOSCA ® isolates including *Rhizobium tibeticum* (16%), *Pseudomonas* spp (14%), *Ensifer meliloti* (2%). Despite commercial claims (Section 2.6.3) that ALOSCA ® is an effective carrier of nodulating bacteria, it failed to perform. A possible explanation is the unevenness of granules and their irregular spread and subsequently that of rhizobia in the seed furrow at sowing. Chances of seed falling in close proximity to the inoculant granule are minimal and thus survival of rhizobia between its introduction to the soil and the development of a legume rhizosphere which it can colonize (Brockwell *et al.* 1995) are compromised. Additionally, ALOSCA ® was developed in Australia where droughts are more severe and temperatures much higher than those experienced in New Zealand. Its properties may therefore be unsuitable or conditions unfavourable for optimum performance.

Findings from this study contradict reports by Rice & Olsen (1988) that lime coated lucerne yields were 85% higher than peat inoculated treatments in the first year. It is possible that the 50 nodules evaluated per treatment were not truly representative of the nodulating bacteria present in the soil. A larger sample size would however have been more expensive, time consuming and laborious given the meticulous and precise nature of the molecular identification methods used.

# Chapter 7

# General discussion and practical implications for farmers

This study aimed to provide best management practices for lucerne establishment in dryland Canterbury from spring to autumn. This was achieved through sowing at different times and examining the effectiveness of commercial inoculants at nodulating plants. In each sowing date, lucerne established successfully following high emergence which was attributed to the use of a fallow. A fallow period conserves moisture and is recommended to avoid transpiration losses for low water holding capacity soils in summer dry environments such as the eastern regions of New Zealand (Avery *et al.* 2008). All seed treatments established adequate plant populations at 10 kg/ha (bare seed). Coated seed however gave the highest populations compared with the rest of the treatments despite equal numbers of seed sown, suggesting the possibility of enhanced seedling growth and survival by the lime coating (Section 2.6.2).

Overall DM yields were variable depending on sowing date. Late spring and summer sown lucerne yielded much more than late summer sown stands (Figure 4.4). This was due to seasonal changes in assimilate partitioning of reserves between the root and shoot of the lucerne plant in addition to a longer growing period and more exposure to light interception. No yield differences were observed from the different inoculation treatments or different established populations. The later suggests yield compensation by lower plant populations through increased number of shoots per plant and individual shoot mass as previously reported by (Teixeira *et al.* 2007a). It also affirms reports by Wynn-Williams (1976) that maximum lucerne yields are attainable at plant populations as low as 30 plants/$m^2$.

This study demonstrated that seedling and regrowth crops yielded differently as a consequence of their varying responses to environmental and biophysical factors. This behaviour was recently observed by Teixeira *et al.* (2011) and was attributed to the existence of a juvenile phase during seedling development (Robertson *et al.* 2002). Physiological processes during the seedling stage were less responsive to environmental factors but were strongly influenced by ontogenic related factors (Section 2.13.1.1). This characteristic of seedling crops results in delayed canopy expansion and reproductive development which

ultimately affects grazing and defoliation. Management of seedlings should therefore be different from that of regrowth crops. In the seedling stage, for example, lucerne must not be frequently grazed but left to grow and accumulate root reserves that will hasten physiological processes in the regrowth stages and improve establishment of stands and their persistence.

Knowledge of biophysical factors and their seasonal effects on lucerne stands is critical for planning and grazing management. In the spring, lucerne growth rates are high and leaf area expansion rates are fast. Canopy closure occurs much sooner and weeds are smothered by the fast growing lucerne plants. Lucerne grazing at this time maximises lamb live weight gains from the increased forage yields prevalent at this time. In autumn, the reverse occurs and above ground growth declines as assimilates are partitioned to the roots for overwintering and spring regrowth. Weed control strategies such as herbicides must be used to reduce weed invasion which ultimately destroy stands.

The inoculation of lucerne in this experiment had no beneficial effects on nodulation, yield or establishment of lucerne. The bare seed control was able to nodulate and fix adequate nitrogen to sustain crop growth and yield as much as lime coated or peat inoculated stands. This indicates the existence and successful infectivity of indigenous rhizobial strains present in the experimental site. The paddock had a previous history of lucerne (Section 3.1) indicating that rhizobia previously inoculated may have remained active in the soil and persisted since the last cropping. These findings question the need to inoculate lucerne, given the ability of un-inoculated bare seed to nodulate and yield as much as treated seed. A recent study on the inoculation of white clover (Lowther & Kerr 2011) questioned the need for its inoculation in New Zealand after observing yield similarities between inoculated and un-inoculated seed. Inoculation may therefore only be useful as a form of insurance (Section 2.5) for farmers otherwise nodulating rhizobia may already be present in adequate populations in the soil.

The variation in nodulating ability of each of the treatments suggests that their properties have a bearing on their ability to transfer viable infective rhizobia to lucerne plants. This is consistent with Stephens & Rask (1971) who reported that formulation inadequacies are the most common barriers to the success of legume inoculants. Genotypic characterisation confirmed the existence of *Ensifer meliloti* in each of the treatments. Coated seed appears to have been the most effective amongst the three followed by peat and lastly ALOSCA ®. Choice of inoculant in future nodulation studies can therefore be selected based on results from DNA analyses performed in this study. The cost of the coated seed may however need to

be put into considering against the percentage advantage it may have compared with peat and ALOSCA ®. Overall the use of a lime coated seed appeared to provide additional benefit to lucerne emergence and established population but not yield or development.

## 7.1 Conclusions

- All seed treatments in the five sowing dates produced adequate plant populations for successful seedling establishment. Coated seed gave the highest initial and established plant population compared with ALOSCA®, peat inoculum and the bare seed control.

- In all sowing dates, seed treatment gave no differences in total dry matter yield, RUE, light interception, leaf appearance rate or reproductive development.

- Autumn sown crops had a lower radiation use efficiency and slower leaf appearance rate than spring sown crops.

- Seedling and regrowth crops showed different responses to biophysical factors (photoperiod) which suggests different management techniques are required.

- Inoculating seed with *Ensifer meliloti* neither improved stand establishment nor increased its likelihood to be the dominant genotype nodulating lucerne in a paddock that had a previous history of growing lucerne.

- Further research is needed to verify at what soil mineral N levels inhibiting N fixation is soils and to quantify N fixing abilities of dominant rhizobial strains found in lucerne nodules.

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# References


Abdolzadeh, A.; Wang, X.; Veneklaas, E.J.; Lambers, H. 2010. Effects of phosphorus supply on growth, phosphate concentration and cluster-root formation in three *Lupinus* species. *Annals of Botany 105*: 365-374.

Allen, E.K.; Allen, O.N. 1958. Biological Aspects of Symbiotic Nitrogen Fixation. Agricultural Experiment Station, Wisconsin, USA. 118 pp.

Altschul, S.F.; Gish, W.; Miller, W.; Myers, E.W.; Lipman, D.J. 1990. Basic local alignment search tool. *Journal of Molecular Biology 215*: 403-410.

Andrews, M. 2011. Nutrient solution composition. Pers. Comm. Received by: Khumalo, Q. Lincoln University.

Angus, J.F.; Gault, R.R.; Good, A.J.; Hart, A.B.; Jones, T.D.; Peoples, M.B. 2000. Lucerne removal before a cropping phase. *Australian Journal of Agricultural Research 51*: 877-889.

Arnold, S.M.; Monteith, J.L. 1974. Plant development and mean temperature in a Teesdale habitat. *Journal of Ecology 62*: 711-720.

Avery, D.; Avery, F.; Ogle, G.I.; Wills, B.J.; Moot, D.J. 2008. Adapting farm systems to a drier future. *Proceedings of the New Zealand Grassland Association 70*: 13–18.

Avice, J.C.; Dily, F.L.; Goulas, E.; Noquet, C.; Meuriot, F.; Volenec, J.J.; Cunningham, S.M.; Sors, T.G.; Dhont, C.; Castonguay, Y.; Nadeau, P.; Bélanger, G.; Chalifour, F.P.; Ourry, A. 2003. Vegetative storage proteins in overwintering storage organs of forage legumes: roles and regulation. *Canadian Journal of Botany 81*: 1198-1212.

Avice, J.C.; Lemaire, G.; Ourry, A.; Boucaud, J. 1997. Effects of the previous shoot removal frequency on subsequent shoot regrowth in two *Medicago sativa* L. cultivars. *Plant and Soil 188*: 189-198.

Bailey, L.D. 1983. Effects of potassium fertilizer and fall harvests on alfalfa grown on the eastern Canadian Prairies. *Canadian Journal of Soil Science 63*: 211-219.

Barlow, N.D.; Goldson, S.L. 1993. A modelling analysis of the successful biological control of *Sitona discoideus* (Coleoptera: Curculionidae) by *Microctonus aethiopoides* (Hymenoptera: Braconidae) in New Zealand. *Journal of Applied Ecology 30*: 165-178.

Baysdorfer, C.; Bassham, J.A. 1985. Photosynthate Supply and Utilization in Alfalfa: A developmental shift from a source to a sink limitation of photosynthesis. *Plant Physiology 77*: 313-317.

Belanger, G.; Castonguay, Y.; Chalifour, F.P.; Dhont, C.; Nadeau, P. 2003. Alfalfa root nitrogen reserves and regrowth potential in response to fall harvests. *Crop Science 43*: 181-194.

Blair, I.D. 1971. Lucerne nodulation failures. *Proceedings of the Lincoln College Farmers Conference 21*: 58-66.

Brockwell, J.; Bottomley, P.J.; Thies, J.E. 1995. Manipulation of rhizobia microflora for improving legume productivity and soil fertility: A critical assessment. *Plant and Soil 174*: 143-180.

Bromfield, E.S.P.; Sinha, I.B.; Wolynetz, M.S. 1986. Influence of location, host cultivar, and inoculation on the composition of naturalized populations of *Rhizobium meliloti* in *Medicago sativa* nodules. *Applied and Environmental Microbiology 51*: 1077-1084.

Brown, H.E. 2004. Understanding yield and water use of dryland forage crops in New Zealand. Ph.D thesis. http://hdl.handle.net/10182/1697. Lincoln University, Christchurch, New Zealand. 288 pp.

Brown, H.E.; Moot, D.J.; Pollock, K.M. 2003. Long term growth rates and water extraction patterns of chicory, lucerne and red clover. pp. 89-97. *In*: Legumes for Dryland

Pastures. Ed. Moot, D.J. Grasslands Research and Practice Series **11**. New Zealand Grassland Association, Christchurch.

Brown, H.E.; Moot, D.J.; Teixeira, E.I. 2005. The components of lucerne (*Medicago sativa*) leaf area index respond to temperature and photoperiod in a temperate environment. *European Journal of Agronomy 23*: 348-358.

Brown, H.E.; Moot, D.J.; Teixeira, E.I. 2006. Radiation use efficiency and biomass partitioning of lucerne (*Medicago sativa*) in a temperate climate. *European Journal of Agronomy 25*: 319-327.

Burton, J.C. 1972. Nodulation and Symbiotic Nitrogen Fixation. pp. 229-246. *In*: Alfalfa: Scence and Technology. Ed. Hanson, C.H. American Society of Agronomy, Wisconsin, USA.

Bushby, H.V.A. 1981. Quantitative estimation of rhizobia in non-sterile soil using antibiotics and fungicides. *Soil Biology and Biochemistry 13*: 237-239.

Caetano-Anollés, G.; Lagares, A.; Favelukes, G. 1989. Adsorption of *Rhizobium meliloti* to alfalfa roots: Dependence on divalent cations and pH. *Plant and Soil 117*: 67-74.

Campbell, M.H.; Swain, F.G. 1973. Effect of strength, tilth and heterogeneity of the soil surface on radicle-entry of surface-sown seeds. *Journal of the British Grassland Society 28*: 41-50.

Carter, J.M.; Tieman, J.S.; Gibson, A.H. 1995. Competitiveness and persistence of strains of rhizobia for faba bean in acid and alkaline soils. *Soil Biology and Biochemistry 27*: 617-623.

Collins, M.; Duke, S.H. 1981. Influence of potassium-fertilization rate and form on photosynthesis and $N_2$ fixation of alfalfa. *Crop Science 21*: 481-485.

Conrad, H.R.; Klopfenstein, T.J. 1988. Role in livestock feeding - greencrop, silage, hay and dehy. pp. 539-551. *In*: Alfalfa and Alfalfa Improvement. Eds. Hanson, A.A.; Barnes, D.K.; Hill Jr., R.R. American Society of Agronomy,Inc., Crop Science Society of America, Inc., Soil Science Society of America, Inc, Madison, Wisconsin.

Corbin, E.J.A.; Brockwell, J.A.; Gault, R.R. 1977. Nodulation studies on chickpea (*Cicer arietinum*). *Australian Journal of Experimental Agriculture 17*: 126-134.

Cox, J.E. 1978. Soils and agriculture of part Paparua County, Canterbury. New Zealand Soil Bureau Bulletin No. **34**. New Zealand Dept of Scientific and Industrial Research, Wellington 127 pp.

Crocker, G.J.; Sheridan, K.P.; Holford, I.C.R. 1985. Lucerne responses to lime and interactions with other nutrients on granitic soils. *Australian Journal of Experimental Agriculture 25*: 337-346.

Cullen, D.W.; Nicholson, P.S.; Mendum, T.A.; Hirsch, P.R. 1998. Monitoring genetically modified rhizobia in field soils using the polymerase chain reaction. *Journal of Applied Microbiology 84*: 1025-1034.

Danso, S.K.A.; Alexander, M. 1973. Survival of two strains of *Rhizobium* in soil. *Soil Science Society of America Journal 38*: 86-89.

de Bruijn, F.J. 1992. Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. *Applied and Environmental Microbiology 58*: 2180-2187.

Demezas, D.H.; Reardon, T.B.; Strain, S.R.; Watson, J.M.; Gibson, A.H. 1995. Diversity and genetic structure of a natural population of *Rhizobium leguminosarum* bv. *trifolii* isolated from *Trifolium subterraneum* L. *Molecular Ecology 4*: 209-220.

Douglas, J.A. 1986. The production and utilization of lucerne in New Zealand. *Grass and Forage Science 41*: 81-128.

Douglas, J.A.; Kinder, J.W. 1973. Production and composition of various luceme and grass mixtures in a semi-arid environment. *New Zealand Journal of Experimental Agriculture 1*: 23-27.

Douglas, M.H.; Brash, D.W.; Barratt, B.I.P.; Keoghan, J.M. 1987. Successful lucerne growing in Inland Otago. *Proceedings of the New Zealand Grassland Association 48*: 193-197.

Fick, G.W.; Holt, D.A.; Lugg, D.D. 1988. Environmental physiology and crop growth. pp. 163-194. *In*: Alfalfa and Alfalfa Improvement. Eds. Hanson, A.A.; Barnes, D.K.; Hill Jr., R.R. American Society of Agronomy,Inc., Crop Science Society of America, Inc., Soil Science Society of America, Inc, Madison, Wisconsin.

Fletcher, A.L. 2000. Thermal time analysis for germination and emergence of temperate pasture species. B.Agr.Sc (Hons) Dissertation. Lincoln University, Canterbury, N.Z. 124 pp.

Frame, J. (Ed.) 2005. Forage Legumes for Temperate Grasslands. Science Publishers, Plymouth, UK. 309 pp.

Frame, J.; Charlton, J.F.L.; Laidlaw, A.S. 1998. Lucerne. pp. 107–179. *In*: Temperate Forage Legumes. C.A.B International, Wallingford.

Gandee, C.M.; Harrison, S.P.; Davies, W.P. 1999. Genetic characterization of naturally occurring *Rhizobium meliloti* populations and their potential to form effective symbioses with lucerne. *Letters in Applied Microbiology 28*: 169-174.

Gillings, M.; Holley, M. 1997. Repetitive element PCR fingerprinting (rep-PCR) using enterobacterial repetitive intergenic consensus (ERIC) primers is not necessarily directed at ERIC elements. *Letters in Applied Microbiology 25*: 17-21.

Glare, T.R.; Ridgway, H.J. 2010. Introduction to techniques in molecular biology. *In*: Paddock to PCR: Demystifying molecular technologies for practical plant protection. Eds. Ridgeway, H.J.; Glare, T.R.; Wakelin, S.A.; O'Callaghan, M. New Zealand Plant Protection Society Inc, Lincoln, N.Z.

Goldson, S.L.; Rowarth, J.S.; Caradus, J.R. 2005. The impact of invasive invertebrate pests in pastoral agriculture: A review. *New Zealand Journal of Agricultural Research 48*: 401-415.

Gomes, N.C.M.; Heuer, H.; Schonfeld, J.; Costa, R.; Mendonca-Hagler, L.; Smalla, K. 2001. Bacterial diversity of the rhizosphere of maize (*Zea mays*) grown in tropical soil studied by temperature gradient gel electrophoresis. *Plant and Soil 232*: 167-180.

Graham, P.H. 1992. Stress tolerance in *Rhizobium* and *Bradyrhizobium* and nodulation under adverse soil conditions. *Field Crops Research 65*: 93-106.

Gürtler, V.; Stanisich, V.A. 1996. New approaches to typing and identification of bacteria using the 16S-23S rDNA spacer region. *Microbiology 142*: 3-16.

Hallmann, J.; Quadt-Hallmann, A.; Mahaffee, W.F.; Kloepper, J.W. 1997. Bacterial endophytes in agricultural crops. *Canadian Journal of Microbiology 43*: 895-914.

Hawkesford, M.; Horst, W.; Kichey, T.; Lambers, H.; Schjoerring, J.; Møller, I.S.; White, P. 2012. Functions of Macronutrients. pp. 135-189. *In*: Marschner's Mineral Nutrition of Higher Plants (3rd Edition). Ed. Marschner, P. Academic Press, San Diego.

Hebb, M.; Richardson, A.E.; Reid, R.; Brockwell, J. 1998. PCR as an ecological tool to determine the establishment and persistence of *Rhizobium* strains introduced into the field as seed inoculant. *Australian Journal of Agricultural Research 49*: 923-934.

Herridge, D.; Gemell, G.; Hartley, E. 2002. Legume Inoculants and Quality Control. pp. 105-115. *In*: Inoculants and Nitrogen Fixation of Legumes in Vietnam. Proceedings of a workshop held in Hanoi, Vietnam 17–18 February 2001. Ed. Herridge, D.Proceedings 109e. ACIAR, Brisbane. http://aciar.gov.au/files/node/2110/pr109echapter13.pdf.

Hoben, H.J.; Somasegaran, P.; Boonkerd, N.; Gaur, Y.D. 1994. Polyclonal antisera production by immunization with mixed cell antigens of different rhizobial species. *World Journal of Microbiology and Biotechnology 10*: 538-542.

Horikawa, Y.; Ohtsuka, H. 1996. Effects of coating and adhesive on the inoculation of *Rhizobium meliloti* to alfalfa (*Medicago sativa* L.) seeds for nodulation and seedling growth. *Grassland Science 41*: 275-279.

Inman-Bamber, N.G.; Smith, D.M. 2005. Water relations in sugarcane and response to water deficits. *Field Crops Research 92*: 185-202.

Iversen, C.E. 1967. Grazing Management of Lucerne. pp. 129-133. *In*: The Lucerne Crop. Ed. Langer, R.H.M. A.H. & A.W. Reed, Wellington.

Janson, C.G. 1972. Cultural weed control in lucerne. *Proceedings of the New Zealand Weed and Pest Control Conference 25*: 92-95.

Jensen, H.L. 1941. Root-nodule formation and nitrogen fixation in leguminous plants. *Australian Journal of Science 3*: 98-99.

Jensen, M.A.; Webster, J.A.; Straus, N. 1993. Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. *Applied and Environmental Microbiology 59*: 945-952.

Johnsson, E.; Berggård, K.; Kotarsky, H.; Hellwage, J.; Zipfel, P.F.; Sjöbring, U.; Lindahl, G. 1998. Role of the hypervariable region in streptococcal M Proteins: Binding of a human complement inhibitor. *The Journal of Immunology 161*: 4894-4901.

Jones, C.A.; Kiniry, K.R. (Eds.) 1986. CERES-Maize: A Simulation Model of Maize Growth and Development. A&M University Press, Texas. 194 pp.

Kain, W.M.; Trought, T.E.T. 1982. Insect pests of lucerne in New Zealand. pp. 49-59. *In*: Lucerne for the 80's. Special Publication No. **1**. Ed. Wynn-Williams, R.B. Agronomy Society of New Zealand, Palmerston North.

Kearney, J.K.; Moot, D.J.; Pollock, K.M. 2010. On-farm comparison of pasture production in relation to rainfall in Central Otago. *Proceedings of the New Zealand Grassland Association 72*: 121-126.

Khaiti, M.; Lemaire, G. 1992. Dynamics of shoot and root growth of lucerne after seeding and after cutting. *European Journal of Agronomy 1*: 241-247.

Kim, T.H.; Ourry, A.; Boucaud, J.; Lemaire, G. 1991. Changes in source-sink relationship for nitrogen during regrowth of lucerne (*Medicago sativa* L.) following removal of shoots. *Functional Plant Biology 18*: 593-602.

Kiwiseed 2010. ALOSCA® Granulated Legume Inoculant. Accessed: 30 March 2011. www.kiwiseed.co.nz/alosca.php

Lee, C.; Smith, D.M. 1971. Influence of nitrogen fertilizer on stands, yields of herbage and protein, and nitrogenous fractions of field-grown alfalfa. *Agronomy Journal 64*: 527-530.

Lowther, W.L.; Kerr, G.A. 2011. White clover seed inoculation and coating in New Zealand. *Proceedings of the New Zealand Grassland Association 73*: 93-102.

Ludecke, T.E. 1967. Sulphur nutrition of lucerne. pp. 143-149. *In*: The Lucerne Crop. Ed. Langer, R.H.M. A.H. & A.W. Reed, Wellington.

Major, D.J. 1980. Photoperiod response characteristics controllling flowering of nine species. *Canadian Journal of Plant Science 60*: 777-784.

Major, D.J.; Hanna, M.R.; Beasley, B.W. 1991. Photoperiod response characteristics of alfalfa (*Medicago sativa* L.) cultivars. *Canadian Journal of Plant Science 71*: 87-93.

Manglitz, G.R.; Ratcliffe, R.H. 1988. Insects and Mites. pp. 671-704. *In*: Alfalfa and Alfalfa Improvement. Eds. Hanson, A.A.; Barnes, D.K.; Hill Jr., R.R. American Society of Agronomy,Inc., Crop Science Society of America, Inc., Soil Science Society of America, Inc, Madison, Wisconsin.

Martin, B.; Humbert, O.; Camara, M.; Guenzi, E.; Walker, J.; Mitchell, T.; Andrew, P.; Prudhomme, M.; Alloing, G.; Hakenbeck, R.; Morrison, D.A.; Boulnois, G.J.; Claverys, J.P. 1992. A highly conserved repeated DNA element located in the chromosome of *Streptococcus pneumoniae*. *Nucleic Acids Research 20*: 3479-3483.

Martínez-Romero, E.; Caballero-Mellado, J. 1996. *Rhizobium* phylogenies and bacterial genetic diversity. *Critical Reviews in Plant Sciences 15*: 113-140.

McLean, E.O. 1971. Potentially beneficial effects of liming: chemical and physical. *Proceedings of the Society of Soil and Crop Science Society of Florida 31*: 189-196.

McWilliam, J.R.; Clements, R.J.; Dowling, P.M. 1970. Some factors influencing the germination and early seedling development of pasture plants. *Australian Journal of Agricultural Research 21*: 19-32.

Michaud, R.; Lehman, W.F.; Rumbaugh, M.D. 1988. World Distribution and Historical Development. pp. 25-91. *In*: Alfalfa and Alfalfa improvement. Eds. Hanson, A.A.; Barnes, D.K.; Hill Jr., R.R. American Society of Agronomy,Inc., Crop Science Society of America, Inc., Soil Science Society of America, Inc, Madison, Wisconsin.

Mills, A.; Smith, M.C.; Lucas, R.J.; Moot, D.J. 2008. Dryland pasture yields and botanical composition over 5 years under sheep grazing in Canterbury. *Proceedings of the New Zealand Grassland Association 70*: 37-44.

Moir, J.L.; Moot, D.J. 2010. Soil pH, exchangeable aluminium and lucerne yield responses to lime in a South Island high country soil. *Proceedings of the New Zealand Grassland Association 72*: 191-196.

Moir, J.L.; Scotter, D.R.; Hedley, M.J.; Mackay, A.D. 2000. A climate driven, soil fertility dependent, pasture production model. *New Zealand Journal of Agricultural Research 43*: 491-500.

Moot, D.J.; Brown, H.E.; Pollock, K.M.; Mills, A. 2008. Yield and water use of temperate pastures in dry environments. *Proceedings of the New Zealand Grassland Association 70*: 51-57.

Moot, D.J.; Brown, H.E.; Teixeira, E.I.; Pollock, K.M. 2003. Crop growth and development affect seasonal priorities for lucerne management. pp. 13 - 18. *In*: Legumes for dryland pastures. Ed. Moot, D.J. Grasslands Research and Practice Series No. **11**. New Zealand Grassland Association, Christchurch.

Moot, D.J.; Robertson, M.; Pollock, K.M. 2001. Validation of APSIM-Lucerne for phenological development in a cool temperate climate. *Proceedings of the Australian Agronomy Conference 10*: http://www.regional.org.au/au/asa/2001/6/d/moot.htm.

Moot, D.J.; W.R., S.; Roy, A.M.; Nicholls, A.C. 2000. Base temperature and thermal time requirements for germination and emergence of temperate pasture species. *New Zealand Journal of Agricultural Research 43*: 15-25.

Moreira, F.M.S.; Gillis, M.; Pot, B.; Kersters, K.; Franco, A.A. 1993. Characterization of rhizobia isolated from different divergence groups of tropical *Leguminosae* by comparative polyacrylamide gel electrophoresis of their total proteins. *Systematic and Applied Microbiology 16*: 135-146.

Mullis, K.B.; Faloona, F.A.; Ray, W. 1987. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods in Enzymology 155*: 335-350.

Munns, D.N. 1967. Nodulation of *Medicago sativa* in soil culture III. Effects of nitrate on root hairs and infection. *Plant and Soil 29*: 33-47.

Musgrave, D.J. 1972. The effect of early cut on crown development of seedling lucerne *Proceedings of the New Zealand Weed and Pest Control Conference 25*: 86-91.

Musgrave, D.J. 1977. Effects of time of sowing on the establishment of oversown legumes. *Proceedings of the New Zealand Grassland Association 38*: 160-166.

Niemann, S.; Dammann-Kalinowski, T.; Nagel, A.; Pühler, A.; Selbitschka, W. 1999. Genetic basis of enterobacterial repetitive intergenic consensus (ERIC)-PCR fingerprint pattern in *Sinorhizobium meliloti* and identification of *S. meliloti* employing PCR primers derived from an ERIC-PCR fragment. *Archives of Microbiology 172*: 22-30.

Niemann, S.; Puhler, A.; Tichy, H.V.; Simon, R.; Selbitschka, W. 1997. Evaluation of the resolving power of three different DNA fingerprinting methods to discriminate among isolates of a natural *Rhizobium meliloti* population. *Journal of Applied Microbiology 82*: 477-484.

Noquet, C.; Avice, J.C.; Ourry, A.; Volenec, J.J.; Cunningham, S.M.; Boucaud, J. 2001. Effects of environmental factors and endogenous signals on N uptake, N partitioning

and taproot vegetative storage protein accumulation in *Medicago sativa*. *Functional Plant Biology 28*: 279-287.

Noquet, C.; Meuriot, F.; Caillot, S.; Avice, J.C.; Ourry, A.; Cunningham, S.M.; Volenec, J.J. 2003. Short-day photoperiod induces changes in N uptake, N partitioning and accumulation of vegetative storage proteins in two *Medicago sativa* cultivars. *Functional Plant Biology 30*: 853-863.

O'Callaghan, M.; Gerard, E.M. 2010 DNA fingerprinting of microbial commumities using PCR-DGGE. pp. 35-53 pp. *In*: Paddock to PCR: Demystifying Molecular Technologies for Practical Plant Protection. Proceedings of an International Symposium held 9 August 2010. Eds. Ridgway, H.J.; Glare, T.R.; Wakelin, S.A.; O'Callaghan, M. 35-53 pp. The New Zealand Plant Protection Society Inc, New Plymouth.

Palmer, T.P. 1982. Weeds in lucerne, why they are there, what harm do they do. pp. 133-135. *In*: Lucerne in the 80's. Special publication No. **1**. Ed. Wynn-Williams, R.B.1. Agronomy Society of New Zealand, New Plymouth.

Palmer, T.P.; Wynn-Williams, R.B. 1976. Relationship between density and yield of lucerne. *New Zealand Journal of Experimental Agriculture 4*: 71-77.

Parle, J.N. 1967. Rhizobia and inoculation of lucerne. pp. 99 - 104. *In*: The Lucerne Crop. Ed. Langer, R.H.M. A.H. & A.W. Reed, Wellington.

Parle, J.N.; Douglas, J.A.; Bailie, T.S. 1973. The survival on seed of *Rhizobium meliloti* from commercial inoculants. *Proceedings of the New Zealand Grassland Association 34*: 253-258.

Pearson, C.J.; Hunt, L.A. 1972. Effects of temperature on primary growth of alfalfa. *Canadian Journal of Plant Science 52*: 1007-1015.

Rice, W.A.; Olsen, P.E. 1988. Soil inoculants for alfalfa grown on moderately acid soil. *Communications in Soil Science and Plant Analysis 19*: 947 - 956.

Richardson, B.J.; Baverstock, P.R.; Adams, M. (Eds.) 1986. Allozyme electrophoresis. Academic Press Australia, North Ryde, NSW. 389 pp.

Robertson, M.J.; Carberry, P.S.; Huth, N.I.; Turpin, J.E.; Probert, M.E.; Poulton, P.L.; Bell, M.; Wright, G.C.; Yeates, S.J.; Brinsmead, R.B. 2002. Simulation of growth and development of diverse legume species in APSIM. *Australian Journal of Agricultural Research 53*: 429-446.

Rodríguez-Navarro, D.N.; Dardanelli, M.S.; Ruíz-Saínz, J.E. 2007. Attachment of bacteria to the roots of higher plants. *FEMS Microbiology Letters 272*: 127-136.

Roughley, R. 1970. The preparation and use of legume seed inoculants. *Plant and Soil 32*: 675-701.

Sackville Hamilton, N.R.; Matthew, C.; Lemaire, G. 1995. In defence of the - 3/2 boundary rule: a re-evaluation of self-thinning concepts and status. *Annals of Botany 76*: 569-577.

Selander, R.K.; Caugant, D.A.; Ochman, H.; Musser, J.M.; Gilmour, M.N.; Whittam, T.S. 1986. Methods of multilocus enzyme electrophoresis for bacterial population-genetics and systematics. *Applied and Environmental Microbiology 51*: 873-884.

Sherrell, C.G. 1984. Sodium concentration in lucerne, phalaris and a mixture of the 2 species. *New Zealand Journal of Agricultural Research 27*: 157-160.

Sims, R.E.H. 1975. Lucerne establishment: Precision versus conventional sowing methods. *Proceedings of the New Zealand Grassland Association 36*: 163-171.

Somers, E.; Vanderleyden, J.; Srinivasan, M. 2004. Rhizosphere bacterial signalling: A love parade beneath our feet. *Critical Reviews in Microbiology 30*: 205-240.

Spent, J.I. 2001. Nodulation in Legumes. Royal Botanic Gardens, Kew. 146 pp.

Stanley, M.; Britton, R.; Christinat, R. 2002. Success with lucerne. PIRSA (Primary Industries and Resources, South Australia), Snowtown, South Australia. 79 pp.

Stephen, R.C. 1964. Grass weeds in lucerne. *New Zealand Journal of Agriculture 108*: 433-441.

Stephen, R.C. 1970. Responses by lucerne and associated volunteer species to applied fertilizers in North Canterbury. *Proceedings of the New Zealand Grasstand Association 32*: 117-125.

Su, C.; Evans, L.J. 1996. Soil solution chemistry and alfalfa response to $CaCO_3$ and $MgCO_3$ on an acidic gleysol. *Canadian Journal of Soil Science 76*: 41-47.

Teixeira, E.I. 2006. Understanding growth and development of lucerne crops (*Medicago sativa* L.) with contrasting levels of perennial reserves. PhD thesis. http://hdl.handle.net/10182/1471. Lincoln University, Canterbury, New Zealand. 274 pp.

Teixeira, E.I.; Brown, H.E.; Meenken, E.D.; Moot, D.J. 2011. Growth and phenological development patterns differ between seedling and regrowth lucerne crops (*Medicago sativa* L.). *European Journal of Agronomy 35*: 47-55.

Teixeira, E.I.; Moot, D.J.; Brown, H.E. 2008. Defoliation frequency and season affected radiation use efficiency and dry matter partitioning to roots of lucerne (*Medicago sativa* L.) crops. *European Journal of Agronomy 28*: 103-111.

Teixeira, E.I.; Moot, D.J.; Brown, H.E.; Fletcher, A.L. 2007a. The dynamics of lucerne (*Medicago sativa* L.) yield components in response to defoliation frequency. *European Journal of Agronomy 26*: 394-400.

Teixeira, E.I.; Moot, D.J.; Brown, H.E.; Pollock, K.M. 2007b. How does defoliation management impact on yield, canopy forming processes and light interception of lucerne (*Medicago sativa* L.) crops? *European Journal of Agronomy 27*: 154-164.

Teixeira, E.I.; Moot, D.J.; Mickelbart, M.V. 2007c. Seasonal patterns of root C and N reserves of lucerne crops (*Medicago sativa* L.) grown in a temperate climate were affected by defoliation regime. *European Journal of Agronomy 26*: 10-20.

Thies, J.E.; Bohlool, B.B.; Singleton, P.W. 1992. Environmental effects on competition for nodule occupancy between introduced and indigenous rhizobia and among introduced strains. *Canadian Journal of Microbiology 38*: 493-500.

Thies, J.E.; Holmes, E.M.; Vachot, A. 2001. Application of molecular techniques to studies in *Rhizobium* ecology: a review. *Australian Journal of Experimental Agriculture 41*: 299-319.

Thompson, J.A. 1983. Production and quality control of legume inoculants. *International Crops Research Institute for the Semi Arid Tropics Information Bulletin No. 17*.

Travis, R.L.; Reed, R. 1983. The solar tracking pattern in a closed alfalfa canopy. *Crop Science 23*: 664-668.

Triplett, E.W.; Sadowsky, M.J. 1992. Genetics of competition for nodulation of legumes. *Annual Reviews of Microbiology 46*: 399-428.

Vance, C.P.; Heichel, G.H.; Phillips, D.A. 1988. Nodulation and symbiotic dinitrogen fixation. pp. 229-257. *In*: Alfalfa and Alfalfa Improvement. Eds. Hanson, A.A.; BArnes, D.K.; Hill Jr., R.R. American Society of Agronomy,Inc., Crop Science Society of America, Inc., Soil Science Society of America, Inc, Madison, Wisconsin.

Varella, A.C. 2002. Lucerne crop responses to continous and intermittent light under artificial and agroforestry regimes. PhD thesis. http://hdl.handle.net/10182/1477. Lincoln University, Canterbury, New Zealand. 268 pp.

Versalovic, J.; Koeuth, T.; Lupski, J.R. 1991. Distribution of repetitive DNA sequences in eubacteria and application of fingerprinting of bacterial genomes. *Nucleic Acids Research 19*: 6823-6831.

Vinuesa, P.; Rademaker, J.L.W.; de Bruijn, F.J.; Werner, D. 1998. Genotypic characterization of *Bradyrhizobium* strains nodulating endemic woody legumes of the Canary Islands by PCR-Restriction fragment length polymorphism analysis of genes encoding 16S rRNA (16S rDNA) and 16S-23S rDNA intergenic spacers, repetitive extragenic

palindromic PCR genomic fingerprinting, and partial 16S rDNA sequencing. *Applied and Environmental Microbiology 64*: 2096-2104.

Ward, C.Y.; Blaser, R.E. 1961. Carbohydrate food reserves and leaf area in regrowth of orchard grass. *Crop Science 5*: 366–370.

Weir, B. 2006. Systemics, specificity and ecology of New Zealand rhizobia. PhD thesis. https://researchspace.auckland.ac.nz/bitstream/handle/2292/394/02whole.pdf?sequence=7. University of Auckland, New Zealand, Auckland. 242 pp.

Weisburg, W.G.; Barns, S.M.; Pelletier, D.A.; Lane, D.J. 1991. 16s ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology 173*: 697-703.

Werner, D.; Newton, W.; Hungria, M.; Loureiro, M.; Mendes, I.; Campo, R.; Graham, P. 2005. Inoculant preparation, production and application. pp. 223-253. *In*: Nitrogen Fixation in Agriculture, Forestry, Ecology, and the Environment. Eds. Werner, D.; Newton, W. Nitrogen Fixation: Origins, Applications, and Research Progress No. **4**. Springer Netherlands.

White, J.G.H. 1967. Establishment of lucerne on acid soils. pp. 105-114. *In*: The Lucerne Crop. Ed. Langer, R.M.H. A.H. & A.W. Reed, Wellington.

White, J.G.H. 1982. Lucerne grazing management for the 80's. pp. 85-90. *In*: Lucerne for the 80's. Special Publication No. **1**. Ed. Wynn-Williams, R.B. Agronomy Society of New Zealand, New Plymouth.

White, J.G.H.; Meijer, G. 1978. Legumes and grasses for dry North Canterbury hill country. *Proceedings of the New Zealand Grassland Association 40*: 170-179.

Whitelaw, J.S. 1975. Growing lucerne for dehydration. *Proceedings of the New Zealand Grassland Association 36*: 80-88.

Willems, A. 2006. The taxonomy of rhizobia: an overview. *Plant and Soil 287*: 3-14.

Williams, J.G.K.; Kubelik, A.R.; Livak, K.J.; Rafalski, J.A.; Tingey, S.V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research 18*: 6531-6535.

Wilson, K.J. 1995. Molecular techniques for the study of rhizobial ecology in the field. *Soil Biology and Biochemistry 27*: 501-514.

Wynn-Williams, R.B. 1976. Effect of sowing date on lucerne emergence, survival, nodulation, and early growth. *New Zealand Journal of Experimental Agriculture 4*: 439-445.

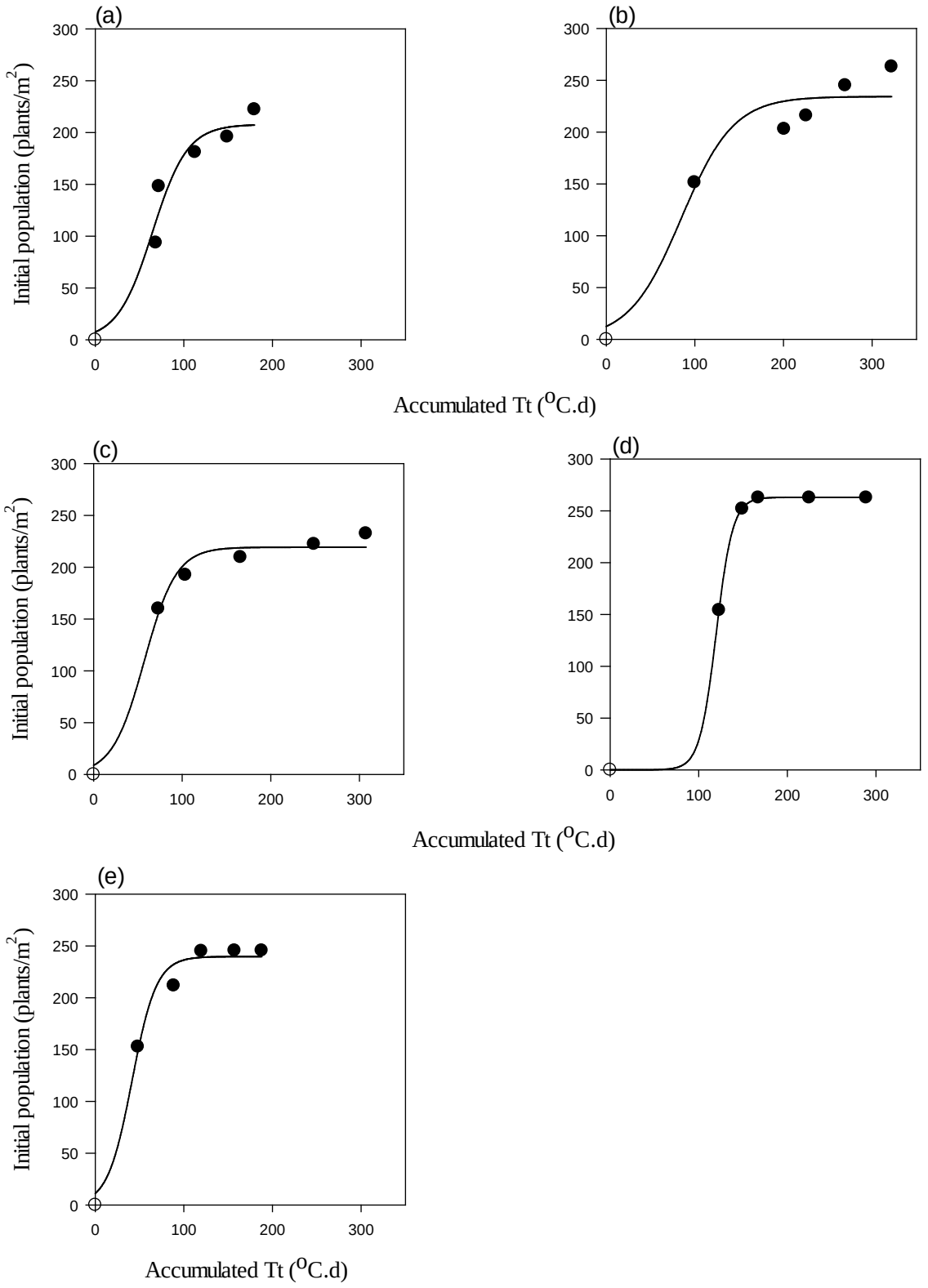
Wynn-Williams, R.B. 1982. Lucerne establishment – conventional pp. 11-20. *In*: Lucerne for the 80's. Special Publication No. **1**. Ed. Wynn-Williams, R.B. Agronomy Society of New Zealand New Plymouth.

Yardin, M.R.; Kennedy, I.R.; Thies, J.E. 2000. Development of high quality carrier materials for field delivery of key microorganisms used as bio-fertilisers and bio-pesticides. *Radiation Physics and Chemistry 57*: 565-568.

Young, J.P.W. 1996. Phylogeny and taxonomy of rhizobia. *Plant and Soil 45*: 45-52.

# Appendices

**Appendix 1 Logistic curves of seedling emergence against days after sowing for 'Stamina 5' seedling lucerne sown on five dates (a-e; (4/10/2010), (4/11/2010), (2/12/2010), (10/01/2011), (7/02/2011)) and treated with a bare seed control (○), ALOSCA® (●), lime coating (▼) or peat inoculant (△) at Lincoln University, Canterbury in 2010.**



**Appendix 2 Nutrient solution composition for incubated seedling lucerne fertigation (Andrews 2011).**

| Nutrients | Molecular Weight | Weight per litre | Molarity (mM) |
|---|---|---|---|
| **Micronutrient Solution (1000 fold)** | | | |
| Iron chloride ($FeCl_2 \cdot 4H_20$) | 198.81 | 0.994g | 5.00 |
| Cobalt chloride ($CoCl_2 \cdot 6H_2O$) | 237.93 | 0.005g | 0.02 |
| Copper sulphate ($CuSO_4 \cdot 5H_2O$) | 249.70 | 0.025g | 0.10 |
| Boric acid ($H_3BO_3$) | 61.83 | 0.309g | 5.00 |
| Sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$) | 241.95 | 0.121g | 0.50 |
| Manganese chloride ($MnCl_2 \cdot 2H_2O$) | 161.87 | 0.162g | 1.00 |
| Zinc sulphate ($ZnSO_4 \cdot 7H_2O$) | 287.53 | 0.029g | 0.10 |
| **Macronutrient Solution (100 fold )** | | | |
| Ammonium nitrate ($NH_4NO_3$) | 80.05 | 0.800g | 10.00 |
| Calcium chloride ($CaCl_2$) | 110.98 | 11.100g | 100.0 |
| Potassium chloride (KCl) | 75.55 | 7.500g | 100.0 |
| Magnesium sulphate ($MgSO_4 \cdot 7H_2O$) | 246.47 | 24.600g | 100.0 |
| Monosodium phosphate ($NaH_2PO_4$) | 119.98 | 12.000g | 100.0 |
| Sodium phosphate ($NaHPO_4$) | 141.96 | 1.400g | 10.0 |